Development of a Highly Sensitive Nucleic Acid Amplification-Based Detection for Human Leptospirosis Infection

Thanyatorn Jiradechbadee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology (International Program)
Prince of Songkla University
2022

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ABSTRACT

The availability of highly sensitive diagnostic tools is crucial for individual screening during the epidemic of leptospirosis. A new approach was evaluated to target *lipL32* gene amplification that combines a conventional quantitative polymerase chain reaction (qPCR) approach and strand displacement isothermal amplification (qPCDR). The gene target used in this study was LipL32 genes. The qPCDR and qPCR reactions carried out with SD polymerase and *taq* polymerase, respectively. The results showed that qPCDR technique presented higher sensitivity than qPCR (can detect 2 copies/µL vs. 20 copies/µL) . Evaluation of qPCDR using pathogenic Leptospira DNA diluted with human DNA samples showed at least ten-fold more sensitive than qPCR assays. Therefore, the qPCDR-based technique developed in this study is a promising approach for pathogenic Leptospira detection and further diagnostic kit development.

ชื่อวิทยานิพนธ์ การพัฒนาเทคนิค nucleic acid amplification-based detection ที่มีความไวสูงเพื่อตรวจหาผู้ป่วยโรคเลปโตสไปโรซิส

ผู้เขียน นางสาวธัญญธร จีระเดชบดี

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บทคัดย่อ

การใช้เครื่องมือการตรวจวัดวินิจฉัยด้วยเทคนิคความไวสูงเป็นสิ่งที่จำเป็นสำหรับขั้นตอนการคัดกรองในช่วงการระบาดของโรคเลปโตสไปโรซิส เพื่อเป็นการพัฒนาเครื่องมือการวินิจฉัยเชื้อก่อโรคด้วยวิธีการเพิ่มจำนวนสารพันธุกรรมโดยใช้เทคนิค quantitative polymerase chain reaction (qPCR) ร่วมกับเทคนิค strand displacement isothermal amplification (qPCDR) มียีนส์เป้าหมายคือ LipL32 ซึ่งในแต่ละปฏิกิริยาจะใช้ชนิดของ DNA polymerase ที่แตกต่างกันดังนี้ เทคนิค qPCDR จะใช้ SD polymerase ในขณะที่ qPCR จะใช้ *taq* polymerase ในปฏิกิริยา จากการศึกษาพบว่าวิธี qPCDR มีความไวต่อปฏิกิริยามากกว่า qPCR (qPCDR สามารถตรวจวัดปริมาณเชื้อเลปโตสไปราจำนวน 2 copies ต่อไมโครลิตรเทียบกับ qPCR 20 copies ต่อไมโครลิตร) นอกจากนี้เมื่อทำการเจือจาง *Leptospira* DNA ที่ก่อโรคกับตัวอย่าง DNA ของมนุษย์พบว่า qPCDR มีความไวสูงกว่า qPCR อย่างน้อยสิบเท่า ดังนั้นการพัฒนาเทคนิค qPCDR ในการศึกษาครั้งนี้มีแนวโน้มเป็นไปในทิศทางบวกและคาดว่าอาจเป็นประโยชน์ต่อการพัฒนาชุดตรวจวัดวินิจฉัยเชื้อก่อโรคเลปโตสไปโรซิสได้ในอนาคต

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µL	Microliter
µg	Microgram
Conc.	Concentration
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
EDTA	Ethylene diamine tetra-acetic acid
g	Gram
ml	Milliliter
PCR	Polymerase chain reaction
qPCR	Quantitative PCR or real-time PCR
rpm	Rotation per minute
PCDR	Polymerase chain displacement reaction
qPCDR	Quantitative PCDR or real-time PCDR
ELISA	Enzyme-linked immunosorbent assay
MAT	Microscopic agglutination test

CHAPTER 1
INTRODUCTION

BACKGROUND AND RATIONALE

Leptospirosis is a zoonosis with a global distribution that is most prevalent in subtropical and tropical climates. It frequently increases seasonally, occasionally in epidemics and is commonly associated with climate change, impoverished urban slum populations, and occupations. The clinical course ranges from moderate to deadly in humans with various medical conditions and clinical signs. Many countries underreport leptospirosis due to a lack of diagnostic laboratory capabilities. Pathogenic Leptospira are bacteria with a long corkscrew structure that is too thin to see with a regular or light microscope. Direct observation of leptospires using darkfield microscopy is imprecise and should be avoided. Isolation of leptospires might take several months and not assist in early diagnosis. Serology diagnoses such as the enzyme-linked immunosorbent assay (ELISA) and microscopic agglutination tests (MAT) are the most commonly used laboratory techniques (Musso and La Scola, 2013). However, MAT needs two different serum samples (acute and convalescent-phase) and distinguishes between IgA and IgG antibodies. The temporal correlation with recent immunization might lead to inaccurate positive results (Heininger *et al.*, 1995). This is especially important when more complicated serology tests, including ELISA, have disadvantages such as a time-consuming/laborious test technique and inadequate sensitivity in bio-recognition of complicated biomolecular entities such as microRNAs.

The polymerase chain reaction (PCR) is an efficient and fast technique for reproducing genetic material. The discovery of thermostable polymerase enzymes has enabled PCR to be processed, reducing the amount of labor required to perform these procedures. PCR offers a wide range of significant and diverse uses in science, health, law, ecology, and archaeology. Although there are certain limits to the technology, such as unintended amplification of contaminated material, PCR has proven indispensable to researchers and has been genuinely revolutionary in the biological sciences. For instance, enzymes used in PCR, *Taq* DNA polymerase high thermostability and strong polymerase activity but no significant strand displacement activity.

An emerging method for clinical diagnosis is polymerase chain displacement reaction (PCDR). PCDR is an approach combining conventional PCR with strand displacement amplification. The technique requires DNA polymerase that performs 5′ to 3′ strand displacement activity and lacks exonuclease activity (Harris *et al.*, 2013, Ignatov *et al.*, 2014). In PCDR, at least two pairs of primers are required for the reaction. The amplification of all primers in the response is initiated simultaneously. As a result, the inner downstream nucleic acid strands are displaced by the outer primers (Ignatov *et al.*, 2014). The displaced nucleic acid strands are employed as additional template strands, thus considerably increasing the sensitivity of the assay.

LITERATURE REVIEW

1. *Leptospira* spp.

1.1 General characteristics of Genus Leptospira

Spirochetes of the genus Leptospira cause leptospirosis. The order *Spirochaetales* includes the family *Leptospiraceae*, which is organized into smaller genera: *Leptospira, Leptonema, and Turneria.* The microagglutination test (MAT) has traditionally separated and categorized *leptospira* spp. into approximately 250 serovars which comprise 35 genomospecies that are divided into three large groups based on genetic relationships comprised of 13 pathogenic (*L. alexanderi, L. alstonii, L. borgpetersenii, L. interrogans, L. kirschneri, L. noguchi, L. santarosai, L. weilii).* 11 saprophytic and 11 intermediate species (Adler and de la Pena Moctezuma, 2010) (Thibeaux *et al.*, 2018). *Leptospires* are gram-negative bacteria corkscrew-shaped and differ from other spirochaetes by the presence of end hooks about 0.1 µm in diameter by 6–20 µm in length (Figure 1a and 1b). They have two periplasmic flagella with polar insertions in the periplasmic space, one connected sub terminally at each end, expanding toward without overlapping. The flagella, which are found inside the spirochete's outer membrane, are essential for cell shape and locomotion (Goldstein and Charon, 1988), (Schmid, 1989). *Leptospira* are aerobic spirochetes possessing a cell wall and cytoplasmic membrane and an outer membrane containing porins that facilitate solute exchange between the periplasmic space and the environment.

Leptospires have a double membrane structure in the cytoplasmic membrane. A peptidoglycan cell wall is inseparably associated with superimposed by an outer membrane (Cullen *et al.*, 2004). Their outer membrane consists of lipopolysaccharide (LPS) with several transmembrane proteins (Haake and Matsunaga, 2010) (Figure 2.). The outer membrane proteins, in general, perform as diffusion barriers and involve in the production of the septum and nutrient uptake for growth (DiRienzo *et al.*, 1978). These membrane proteins have been demonstrated to evaluate the amount of virulence against host mechanisms and confront host defense mechanisms since they are exposed to the host environment directly in the exterior (Cullen *et al.*, 2004). For instance, Lipoprotein L32 (LipL32) (Yang *et al.*, 2002), *Leptospira* immunoglobulin-like proteins (Lig) (Lin *et al.*, 2008), *Leptospira* endostatin-like proteins (Len) (Stevenson *et al.*, 2007) (Matsunaga *et al.*, 2003) and *Leptospira* OmpA-like lipoprotein (Loa22) (Ristow *et al.*, 2007).

Due to their virulent characteristics, they have been reported in the literature. Many procedures were used to separately isolate the proteins as mentioned above using different surfactants, allowing them to be categorized into five primary protein groups (Figure 3.) (Auran *et al.*, 1972) (Sasaki *et al.*, 2018) (Thoduvayil *et al.*, 2020).

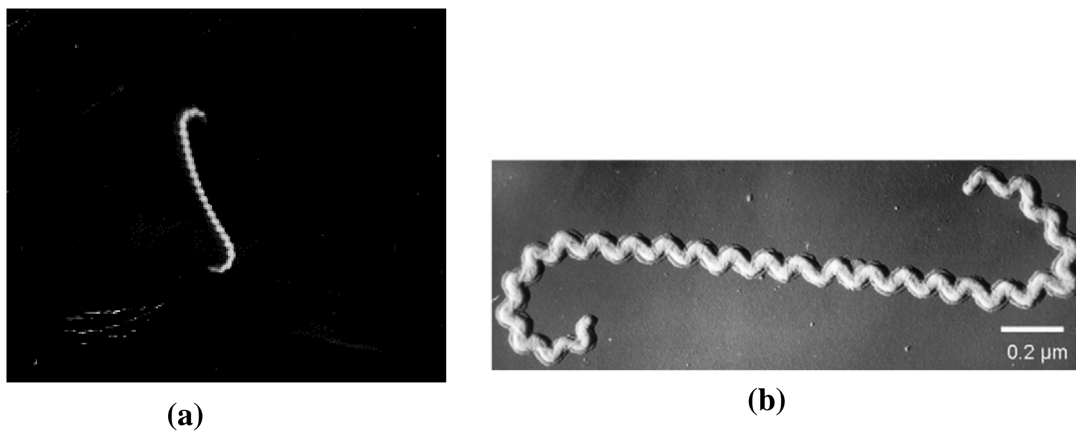

Figure 1. **(a)** Scanning electron microscope morphology of representative cells of a novel, the Spirochete *Leptospira species*. **(b)** photomicrographs of *Leptospira* spp. (Picardeau *et al.*, 2001).

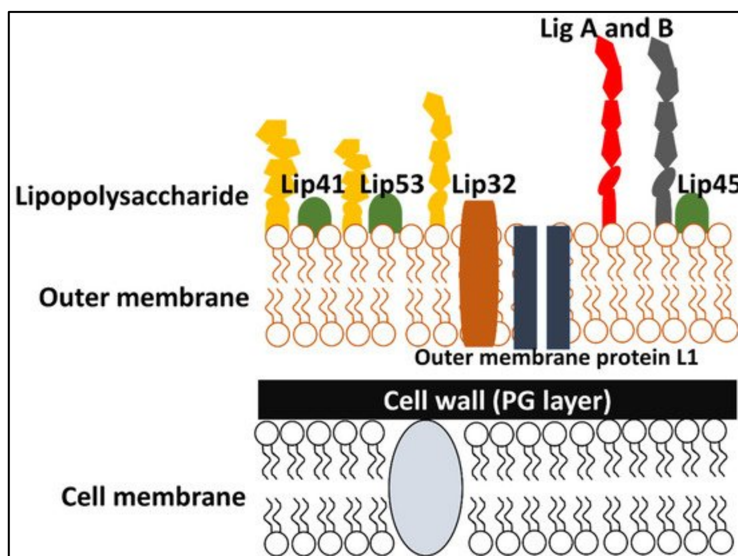

Figure 2. The various proteins found on the outer membrane (Haake and Matsunaga, 2010)

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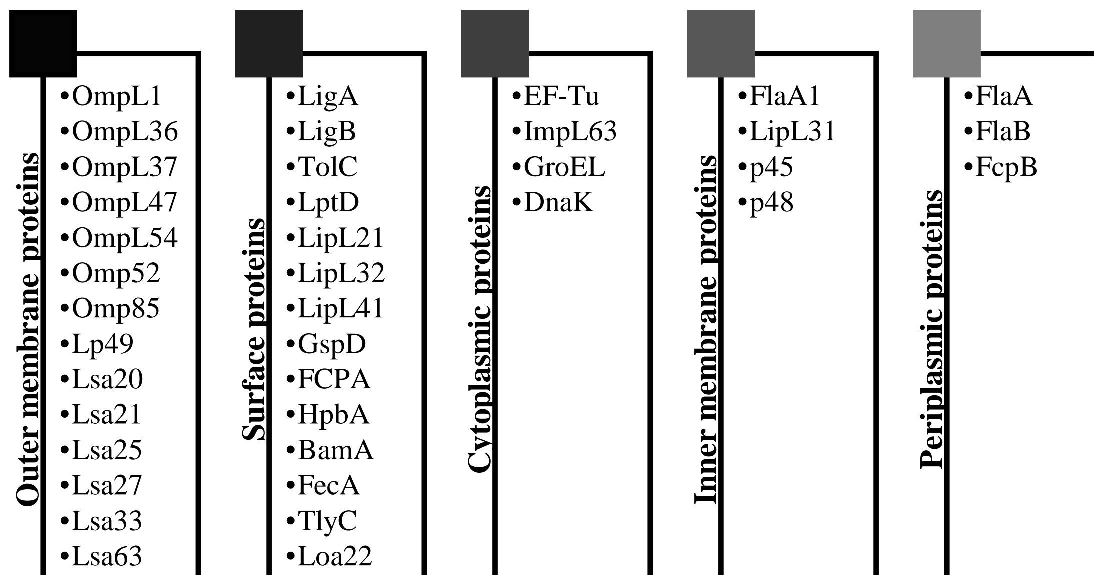


Figure 3. A representation of *leptospiral* proteins is classified into five major classes (Haake and Matsunaga, 2010).

Lipoprotein L32 (LipL32)

Lipoprotein L32 is 32-kDa lipoprotein and the most prominent protein in the leptospiral protein profiles. It has a polyaspartate (polyD) region with a cluster of seven aspartate residues in an eight-amino-acid phase with the sequence ^{142}DDDDDGDD^{149}. The LipL32 polyD region is found in the center of the protein and is evolutionarily conserved among the *leptospira* superfamily of LipL32 proteins (Haake *et al.*, 1998).

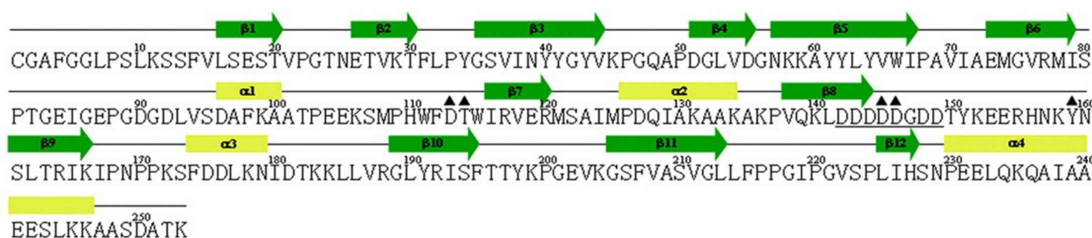


Figure 4. The secondary structural components and amino acid sequence of LipL32. Green and yellow colors are used for the β-strands and α-helices, respectively. The polyD sequence is emphasized (^{142}DDDDDGDD^{149}). The amino acids Asp^{113}, Thr^{114}, Asp^{145}, Asp^{146}, and Tyr^{159} are marked (Tung *et al.*, 2010).

It has a single tag at its N-terminus and a specialized lipid-based modification at its Cysteine residue, highly conserved within pathogenic *leptospira* species (Tung *et al.*, 2010). This major outer membrane protein (MOMP) could be solubilized by extracting the outer membrane with the nonionic detergents Triton X-100 and Triton X-114 (Zuerner *et al.*, 1991). The virulence of Lipl32 is associated with the host's innate immunity because it triggers an inflammatory reaction in the host. LipL32 also functions as a hemolysin, causing proinflammatory cytokines to be released via several toll-like receptors (TLR) signaling pathways (Wang *et al.*, 2012). TLR2 and TLR4 are the two kinds of TLRs implicated in leptospirosis, although TLR2 has a significant pathogenic relevance since it interacts directly with LipL32 (Yang *et al.*, 2006). They also discovered that the calcium-binding cluster, which is made up of a number of essential residues including such aspartic acid (Asp), threonine (Thr), and tyrosine (Tyr), all of which are found on *LipL32*, is responsible for maintaining *LipL32* conformation for suitable TLR2-mediated inflammation in host renal cells (Lo *et al.*, 2013). For the leptospirosis diagnostic, LipL32 is a target gene for multiplex polymerase chain reaction (PCR), which increases the sensitivity and specificity of leptospiral infection diagnosis (Ahmed *et al.*, 2012).

1.2 Taxonomy and classification

1.2.1 Serological classification

Leptospires are bacteria that can be divided into pathogenic and saprophytic leptospires. The pathogenic one is capable of causing disease in human, while saprophytic is free-living and commonly incapable of causing disease. Saprophytes are not predicted to affect illness. They are commonly detected in cultures obtained from clinical samples, but their relevance is unknown. Their primary role in medical microbiology is as contaminants in materials that are ostensibly sterile or at the very least saprophyte-free. Saprophytic species of *Leptospira* include *L. biflexa, L. meyeri, L. yanagawae, L. kmetyi, L. vanthielii and L. wolbachii,* and contain more than 60 serovars. Pathogenicity to animals, growth, and other tests distinguish pathogenic and saprophytic leptospires. The low-temperature test employs that because the minimum growth temperature ranges from 13 to 15°C for pathogenic, whereas saprophytes must be kept at 5-10°C. Different serovars can demonstrate different host specificities and may not with a specific clinical manifestation of infection. Therefore, proper identification and categorization of *Leptospira* spp. is crucial for epidemiological and public health surveillance.

1.2.2 Genotyping classification

The phenotypic classification of leptospiral has been substituted with several genomospecies, including serovars of *L. interrogans* and *L. biflexa.* Genetic heterogeneity was revealed, and DNA hybridization investigations identified Leptospira genomospecies. Later, the genomospecies *L. kirschneri* was established. The Centers for Disease Control (CDC) classified *Leptospira* spp. into 16 genomospecies. Recently, there are

five new genomospecies, one of which was called *L. alexanderi*, after an exhaustive examination of several hundred strains. Since then, a new species, *L. fainei*, has been described, with a new serovar, hurstbridge (Perolat *et al.*, 1998). DNA hybridization tests have also verified the monospecific genus Leptonema's phylogenetic analysis (Postic *et al.*, 2000).

Although recent investigations suggest that more research is needed, multilocus enzyme electrophoresis results support the genotypic classification of leptospires. *Leptospira* has different genomospecies than the preceding two species (*L. interrogans* and *L. biflexa*), and pathogenic and nonpathogenic serovars coexist within the same species. As a result, neither the serogroup nor the serovar consistently predicts the Leptospira species (Table 1). Furthermore, current research has incorporated several strains of specific serovars, revealing genetic variability within serovars (Table 2) (Feresu *et al.*, 1999).

Table 1 Genomospecies of *Leptospira* and distribution of serogroups

Species	Serogroups[b]
L. interrogans	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin
L. noguchii	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona
L. santarosai	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri
L. meyeri	Ranarum, Semaranga, Sejroe, Mini, Javanica
L. wolbachii[c]	Codice
L. biflexa[c]	Semaranga, Andamana
L. fainei	Hurstbridge
L. borgpetersenii	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
L. kirschneri	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae,
L. weilii	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
L. inadai	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica
L. parva[c]	Turneria
L. alexanderi	Manhao, Hebdomadis, Javanica, Mini

Table 1 Genomospecies of *Leptospira* and distribution of serogroups (continued)

Serogroup	Genomospecies
Andamana	*L. biflexa*
Australis	*L. interrogans, L. noguchii, L. borgpetersenii, L. kirschneri*
Autumnalis	*L. interrogans, L. noguchii, L. santarosai, L. borgpetersenii, L. kirschneri*
Ballum	*L. borgpetersenii*
Bataviae	*L. interrogans, L. noguchii, L. santarosai, L. borgpetersenii, L. kirschneri*
Canicola	*L. interrogans, L. inadai, L. kirschneri*
Celledoni	*L. weilii, L. borgpetersenii*
Codice	*L. wolbachii*
Cynopteri	*L. santarosai, L. kirschneri*
Djasiman	*L. interrogans, L. noguchii, L. kirschneri*
Grippotyphosa	*L. interrogans, L. santarosai, L. kirschneri*
Hebdomadis	*L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. kirschneri, L. alexanderi*
Hurstbridge	*L. fainei*
Icterohaemorrhagiae	*L. interrogans, L. weilii, L. inadai, L. kirschneri*
Javanica	*L. weilii, L. santarosai, L. borgpetersenii, L. meyeri, L. inadai, L. alexanderi*
Louisiana	*L. interrogans, L. noguchii*
Lyme	*L. inadai*
Manhao	*L. weilii, L. inadai, L. alexanderi*
Mini	*L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. meyeri, L. alexanderi*
Panama	*L. noguchii, L. inadai*
Pomona	*L. interrogans, L. noguchii, L. santarosai, L. kirschneri*
Pyrogenes	*L. interrogans, L. noguchii, L. weilii, L. santarosai, L. borgpetersenii*
Ranarum	*L. interrogans, L. meyeri*
Sarmin	*L. interrogans, L. weilii, L. santarosai*
Sejroe	*L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. meyeri*
Semaranga	*L. meyeri, L. biflexa*
Shermani	*L. noguchii, L. santarosai, L. inadai*
Tarassovi	*L. noguchii, L. weilli, L. santarosai, L. borgpetersenii, L. inadai*

Table 2 Leptospiral serovars found in multiple species

Serovar	Species
bataviae	*L. interrogans, L. santarosai*
bulgarica	*L. interrogans, L. kirschneri*
grippotyphosa	*L. kirschneri, L. interrogans*
hardjo	*L. borgpetersenii, L. interrogans, L.meyeri*
icterohaemorrhagiae	*L. interrogans, L. inadai*
kremastos	*L. interrogans, L. santarosai*
mwogolo	*L. kirschneri, L. interrogans*
paidjan	*L. kirschneri, L. interrogans*
pomona	*L. interrogans, L. noguchii*
pyrogenes	*L. interrogans, L. santarosai*
szwajizak	*L. interrogans, L. santarosai*
valbuzzi	*L. interrogans, L. kirschneri*

Leptospirosis and epidemiology

Leptospirosis is the most prevalent zoonosis globally, especially in poor tropical countries during the rainy season (Ko *et al.*, 2009). It is a pathogenic member of the genus *Leptospira* that affects humans and animals. Rats and other rodents are the most common causes of infection. An infected animal might be asymptomatic while still shedding germs in its urine. Humans usually become infected through contact with urine contaminated soil, mud, or water with infected animal tissue. The animal carriers might be wild or domestic animals, specifically rodents and small marsupials, cattle, pigs, and dogs (Lecour *et al.*, 1989) (Everard *et al.*, 1995) (Figure 5.). Agricultural occupations such as farmers, sewer workers, miners, fisheries, and even meat workers have been at a high risk of infection in areas with poor sanitation. An overview of the leptospirosis transmission and pathogenesis framework is shown in figure 6.

Leptospires usually enter the host through abrasions, integument, conjunctiva, mucous membrane, or even sexual organ. They are alive in the proximal renal tubules of the kidneys of carriers (Figure 7.). They are excreted in the urine for a few weeks to many months. The bacteria always required chemotaxis mechanisms for adhesion and transmembrane passages (Thompson and Manktelow, 1986). This led to contaminated soil, surface water, streams, and rivers. Leptospires cannot survive in acid urine, but they might stay in alkaline urine. As a result, herbivores and animals whose diets produce alkaline urine are more significant shedders than those that have acid urine (Bharti *et al.*, 2003).

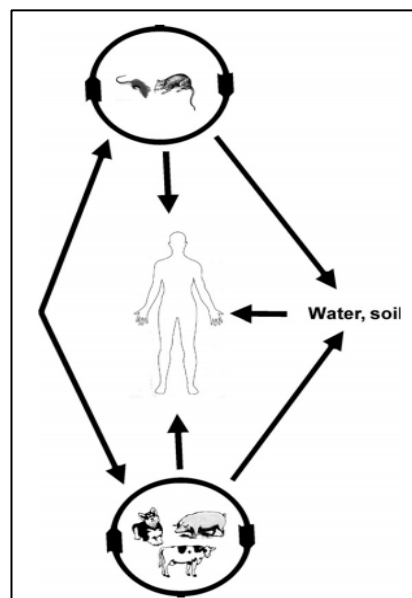


Figure 5. Epidemiology of leptospirosis in animals and humans (Adler and de la Pena Moctezuma, 2010)

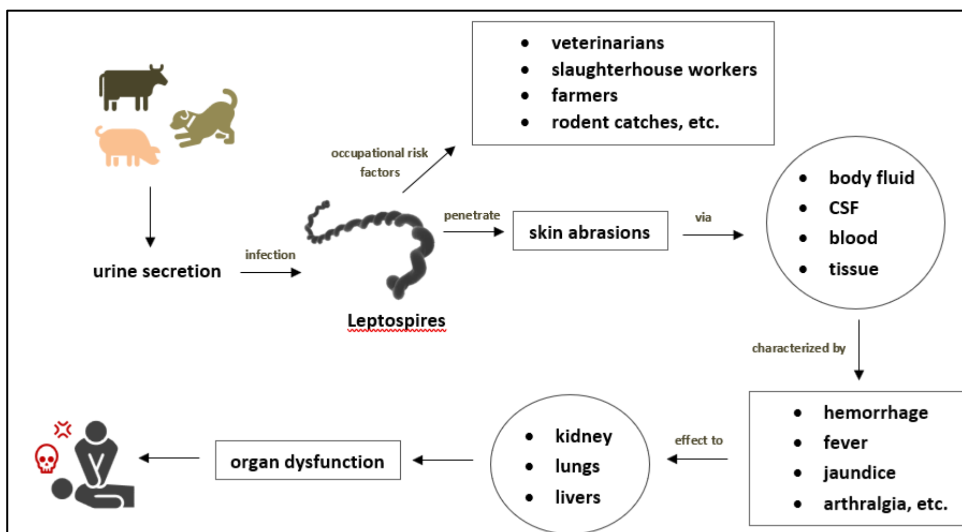


Figure 6. An overview of the leptospirosis transmission and pathogenesis framework

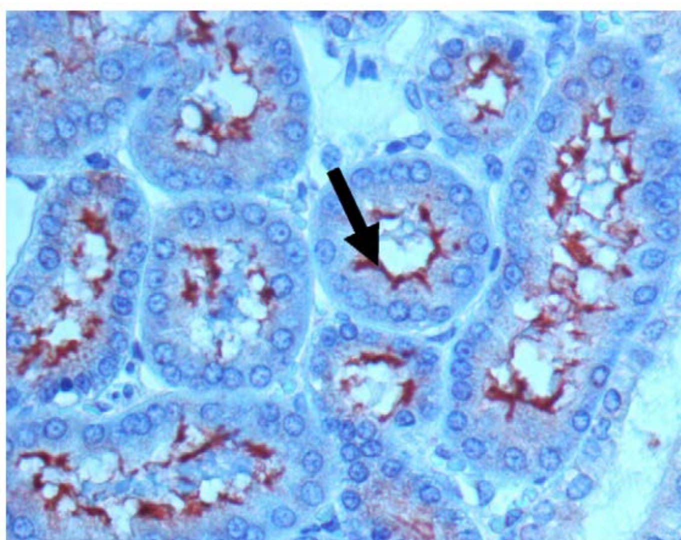


Figure 7. Infected hamster kidney immunohistopathology. The proximal renal tubules are lined by leptospires stained with specific antiserum (arrow) (Adler and de la Pena Moctezuma, 2010)

2. Pathogenesis and clinical manifestations

The manifestation of leptospirosis might vary from one day to at least four weeks after exposure (Manev *et al.*, 1987) (Borer *et al.*, 1999). Fever, headache, arthralgia, erythema, cephalgia, and myalgia are common symptoms. There are two phases of illness (Figure 8). The septicemic stage is the first, often known as the acute phase. Leptospira are detected in the circulation at this phase which they reproduce in the absence of particular antibodies (leptospiremia) and disseminate to numerous organs during a period of three to ten days (Agampodi *et al*., 2012) (Bharti *et al.*, 2003) (Coutinho *et al.*, 2014) (Ko *et al.*, 2009).

The second phase, the immunological phase, generally begins in the second week after symptoms and lasts for a few months. Leptospires are eliminated from the circulation during this phase. The antibodies are elevated at this phase, and leptospires are no longer present in the circulation (Ko *et al*., 2009) (Levett, 2001). According to animal studies, most tissues are invaded, especially in kidneys where leptospires allocate into renal tubules away from circulating specific antibodies, particularly hamsters (Coutinho *et al.*, 2014). The second phase, the immunological phase, generally begins in the second week after symptoms and lasts for a few months. Leptospires are eliminated from the circulation during this phase. (Silva *et al.*, 1995).

When leptospirosis is untreated during the acute phase, leptospires in the circulation can be translocated to the host tissues. They multiply and become highly invasive, secreting vast amounts of cell membrane damaging enzymes. As a result, the condition gradually worsens. This stage is commonly referred to as Weil's illness, a severe type of leptospirosis. The late phase, leptospirosis, and icteric phase are other names (Lau *et al.*, 2018), (Asensio-Sanchez *et al.*, 2018). Additionally, patients who are untreated for a long time might develop fatal hepatic manifestations. Severe leptospirosis can cause liver failure, renal failure, and respiratory shock, among other things.

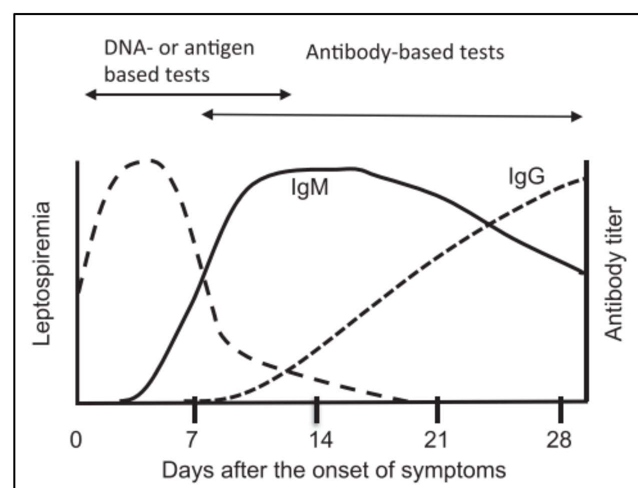


Figure 8. Leptospiral infection kinetics in the bloodstream. The infection causes leptospiraemia in the first few days after exposure, followed by leptospires migration to target organs (Agampodi *et al.*, 2012).

3. Leptospirosis diagnosis

Diagnosis is difficult in the early stages of leptospirosis because of the clinical characteristics. Because of various clinical symptoms, leptospirosis is often misdiagnosed as scrub typhus, dengue fever, or even malaria. (Suttinont *et al.*, 2006) (Table 3). Clinical signs indicating Weil's illness are present in a procedure and those clinical features are apparent. The severity of the disease differs depending on the individual and infecting strain. At the same time, clinical characteristics given by patients cannot be used to confirm the disease. The laboratory diagnosis may be a reasonable choice for the diagnostic test. To ensure the presence of infection, several diagnostic methods have been developed to confirm leptospirosis diagnosis. In the present circumstance, the diagnosis of leptospirosis is principally based on serological, indirect, and direct diagnostic methods. The detection of specific antibodies against various leptospiral antigens is usually the basis of previous studies. Because leptospires have long doubling periods in culture and require a long time to grow, leptospirosis is generally diagnosed based on serological testing.

Table 3. Differential diagnosis of leptospirosis (Plank and Dean, 2000)

Endemic areas
Dengue
Rickettsioses (Q fever, typhus)
Malaria
Pulmonary tuberculosis
Viral hepatitis
Bacterial or viral meningitis
Influenza
Brucellosis
Ehrlichiosis
Tularemia
Syphilis
HIV
Sepsis
Yellow fever
Non-endemic areas
Pyelonephritis/urinary tract infection
Overwhelming adenovirus infection
Acute abdomen
Gastroenteritis
Atypical pneumonia
In travelers, viral hemorrhagic fever (dengue, Lassa, Congo-Crimean, Rift Valley, Ebola Sudan and Zaire, and Marburg)
In lung-renal syndrome, as connective tissue disorders or vasculitis

3.1 Microscopic Agglutination Test (MAT)

MAT technique is the gold standard and most widely used for diagnosing leptospirosis. A positive sample would test different serum dilutions to determine the MAT titer. A four-fold increase in MAT antibody titer indicates *leptospira* spp. infection (Chirathaworn *et al.*, 2014). In addition, the serum from patients might react with a different serovar. Because MAT is a sophisticated test, executing it with many samples would be challenging.

Nonetheless, it would be useless in the early stages of the disease since the antibodies against leptospires are absent, and the CSF level will be incredibly low (Bajani *et al.*, 2003) (Budihal and Perwez, 2014). Furthermore, The MAT is difficult to standardize, and the particular reason circumstance of the requirement for living organisms to maintain antigen levels causes harm to laboratory staff. Then, the accuracy of nucleic-acid diagnostics and rapid antibody-based assays as safe diagnostic techniques for laboratory staff are requested.

3.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA may be done with little training and generally provides outcomes in 2–4 hours. They used *leptospiral*-specific IgM and IgG from the serum of individuals infected with various *leptospiral* serovars. Even with low antigen titers in the patients' serum, leptospires generated specific IgM and IgG. Only a few individuals generated IgG agglutinins, although they all produced IgM agglutinins. The specificity of the antisera used to prepare the conjugates was confirmed by immunodiffusion and immulectrophoretic against purified human IgM and IgG immunoglobulins (Desakorn *et al.*, 2012) (Figure 9). Antigens have included recombinant surface proteins and even lipoproteins. Antigens might not always identify the variety of circulating strains, and the sensitivity of these tests is often low (Levett, 2001), (McBride *et al.*, 2005). However, the ELISA method cannot provide concrete evidence of serological diagnosis; laboratory confirmation by MAT, PCR, or at least cis is still required.

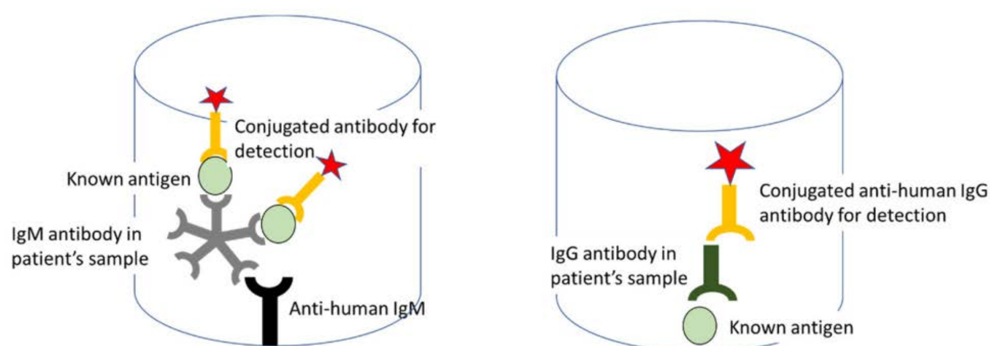


Figure 9. Antibodies against leptospirosis (IgM and IgG) used to diagnose leptospirosis (Desakorn *et al.*, 2012).

3.3 Flow cytometry (FCM)

Because of its high sensitivity to the size and form of leptospires, it has been used to identify leptospirosis (Tzur *et al.*, 2011). The scattering parameters play an essential role in this technique, including ward scatter (FSC) and side scatters (SSC). FSC is correlated to cell size and the outer membrane's optical refraction index, whereas SSC is related to bacterial granularity. (Adan *et al.*, 2017). After performing the agglutination reaction between the antigen and antibody of a particular serovar type in Leptospira, the diagnostics may be accomplished by analyzing the light scattering patterns. Because advanced flow cytometers have the resolution to identify and monitor particles with a diameter less than 0.5 µm, analysis is possible. (Headland *et al.*, 2014).

3.4 Dipstick assay

Non-enzymatic reactions using a stabilized anti-human IgM dye conjugate detect IgM antibodies with a sensitivity equivalent to IgM-ELISA (Hatta *et al.*, 2000). It is a complicated technique for rapidly detecting and diagnosing individuals with leptospirosis. This technique does not require special laboratory equipment or well-trained personnel (Hatta *et al.*, 2000) (Gussenhoven *et al.*, 1997). The samples were evaluated using a dipstick with two horizontal bands. The bottom band contained broadly reactive specific antigens, and the upper band had antihuman IgM antibodies, which served as an internal control (Figure 10).

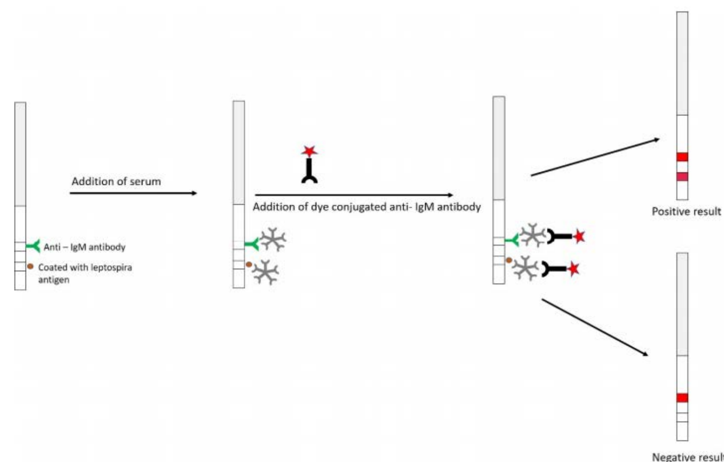


Figure 10. Illustration depiction of dipstick assay (Hatta *et al.*, 2000)

3.5 Staining techniques

It is used in histopathological staining and Warthin-Starry stain is currently widely used (Azizi *et al.*, 2014). Secondary antibodies were labeled with enzymatic or metallic markers in this procedure. For leptospires staining in clinical specimens, phosphatase, peroxidase, or even metallic gold-tagged antibodies can be applied in various patterns. Importantly, this method works well together form with formalin-fixed tissue and may be utilized when the quantity of leptospires is minimal (Budihal and Perwez, 2014).

3.6 Culture techniques

In general, Culture is hardly used in clinical laboratories due to requiring a long-term culture, the doubling time is about 6 to 8 hours, and the entire culture can take over 3 months to grow. Furthermore, because leptospires are highly infectious organisms, they must be handled with utmost caution by trained individuals. This technique, however, continues to play a pivotal role in the study of epidemics and epidemiology for pathogenesis investigations. The advancement of leptospires growth and recovery rates are pretty, very low in the cultivation medium. Isolating the leptospires from cerebrospinal fluid (CSF), urine, dialysis fluid, or blood is the standard, but culture is not routinely. Urine is the most reliable body fluid to study because it contains leptospires from clinical symptoms until the third week of infection. Blood cultures might be negative due to being too collected the sample early or too late. Leptospires might not be detected in the blood until 4 days after the onset of symptoms (7 to 14 days after exposure). When the immune system is activated, blood cultures might become negative again. Culture of blood and CSF during the first week of illness could be helpful for the diagnosis confirmation.

The disease is diagnosed by culturing and isolating Leptospira cells with either rabbit serum (Fletcher's medium) or bovine serum albumin and fatty acids (Ellinghausen-McCullough-Johnson-Harris: EMJH) medium (Johnson and Harris, 1967). Inoculating 1 to 5 drops (100 to 200 µL) of whole blood into EMJH and culturing at 30°C is the standard technique. Antibodies, antibiotics, hemoglobin, and other blood component factors should not be used to suppress leptospire growth. (Adler and de la Pena Moctezuma, 2010). The antibiotics for instance, rifampicin, neomycin, actidione can be used to isolate bacteria from contaminated specimens selectively (Miraglia *et al.*, 2009). Primary cultures were carried out in a semisolid (0.2% agar) medium in which 5-fluorouracil was added as a selective agent. The most often utilized EMJH medium is oleic acid-albumin. It is made up of an essential medium including several enrichment factors such as ammonium chloride (NH_4Cl), thiamine, disodium phosphate (Na_2HPO_4), monopotassium phosphate (MKP), and also Tween 80 and albumin (Ellinghausen and McCullough, 1965).

Samples from a suspected patient, generally urine or blood, are streaked onto a culture flask containing fluid media, the most widely utilized of which is EMJH's oleic acid-albumin medium. It comprises ammonium chloride, thiamine, disodium phosphate, and monopotassium phosphate in a primary media including Tween 80 and albumin (Ellinghausen and McCullough, 1965) (Miraglia *et al.*, 2009). The cultures were examined for signs of growth for instance turbidity, haze, or a ring of growth (Dinger's ring) (Figure 11). and by using

darkfield illumination initially on days one, three and five, followed by seven to ten days intervals up to 6 weeks (Bhatia *et al.*, 2015). It has limitations in that it takes a long time to divide (estimated doubling time is 6–8 hours) and the entire culture can take nearly three months to grow. However, leptospires are extremely infectious organisms, and they must be handled with extreme caution by trained professionals.

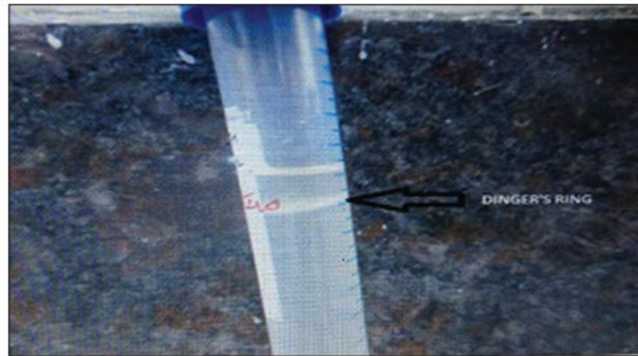


Figure 11. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium showing growth of *Leptospira inadai* as Dinger's ring (Bhatia *et al.*, 2015)

Microscopy techniques are used to observe leptospires in culture. Darkfield microscopy helps easily detect the organism, which appears as a thin, coiled, and motile organism in the blood or urine of a leptospirosis patient (Chandrasekaran and Gomathi, 2004) (Figure 12). In the case of the clinic samples have a lower number of leptospires. It could be concentrated by using centrifugation or high-speed vacuum. However, this technique still has both false positive and false negative, which is probably caused by the experienced of laboratory personnel.

Figure 12. *Leptospira inadai* under a dark field microscope (×400) (Bhatia *et al.*, 2015)

3.7 Polymerase chain reaction (PCR)

More recently, the discovery of real-time PCR (qPCR) has revolutionized infectious diseases molecular diagnostics. It is an *in vitro* method for amplifying specific target DNA sequences by more than 10^6 fold (Saiki *et al.*, 1988). It is widely used to diagnose infectious diseases caused by fastidious or slowly growing bacteria, for example, *Mycobacterium leprae* (Woods and Cole, 1989), *Mycobacterium tuberculosis* (De Wit *et al.*, 1990) , *Treponema pallidum* (Hay *et al.*, 1990), and *Borrelia burgdorferi* (Rosa and Schwan, 1989). Furthermore, it would be quicker and more sensitive than culture. As a result, PCR can confirm infection quicker than serological testing. QPCR provides several advantages over traditional PCR: it is simple to use and consumes less effort, it has less variability and contamination, it enables online monitoring, and it does not require post-reaction investigations (Ahmed *et al.*, 2009) (Picardeau *et al.*, 2014).

4. Polymerase chain displacement reaction (PCDR)

Currently, traditional PCR employs a pair of primers, comprising a forward and reverse primer, to generate a maximum of a two-fold amplicon for each amplification cycle. Polymerase chain displacement reaction (PCDR) is a variation of PCR that employs more than one pair of primers. Several pairs of primers are positioned on either side of the target region of interest. When the outer and inner primers are extended, the outer primer's extended strand causes the internal primer's extended strand to be displaced (Figure 13). After each amplification cycle, PCDR allows producing more than two-fold amplicons. As a consequence, it has a higher sensitivity and faster assay speed. Amplification tests with a better sensitivity would be helpful in diagnostic applications when the DNA target has a low initial copy number (Al-Soud and Radstrom, 2001).

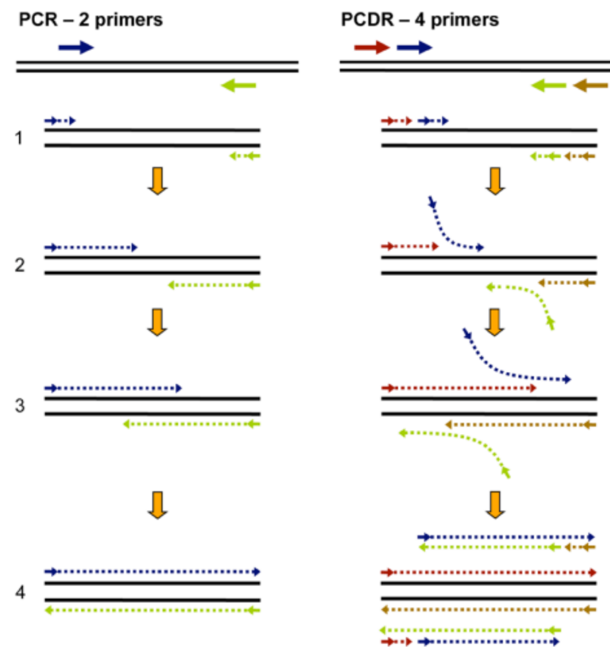


Figure 13. Schematic showing the mechanism of PCDR (Harris *et al.*, 2013)

Nowadays, DNA polymerases derived from thermophilic *Bacillus species*, such as *Bst* DNA polymerase and its derivatives, are utilized in loop-mediated amplification (LAMP) (Kiefer *et al.*, 1997). Nonetheless, at temperatures over 70°C, they become unstable. PCR enzymes, such as *Taq* DNA polymerase, have a high thermostability but lack strand displacement activity, making them inappropriate for isothermal amplification techniques like LAMP (Harris *et al.*, 2013). In case of PCDR require a DNA polymerase that combines the high thermostability of *Taq* DNA polymerase and the strong strand displacement activity of *Bst* DNA polymerase. The polymerase utilized in PCDR is a modified *Taq* DNA polymerase that incorporates elements including high thermostability (up to 93–94°C), 5'-3' polymerase activity, 5'-3' strand displacement activity, and an absence of exonuclease activity, avoiding degradation of the inner primer extension product in the process. Those properties belong to SD DNA polymerase. This polymerase, a strand displacement activity is advantageous in PCR. Furthermore, SD DNA polymerase was considerably more effective than Taq polymerase in overcoming issues with amplifying of DNA templates with complex structures (GC-rich sequences or hairpin structures) (Figure 14). In addition, in the PCDR amplification with four primers, SD polymerase produced considerably greater product levels compared with PCR that contained only two primers (Figure 15 and 16) (Ignatov *et al.*, 2014).

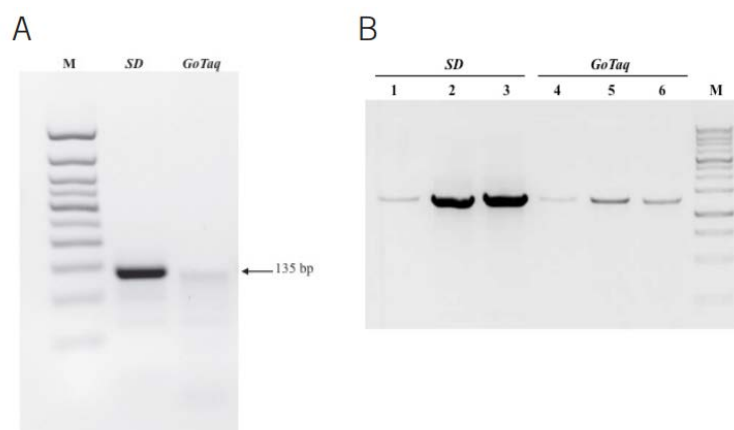


Figure 14. PCR amplification with SD and *Taq* DNA polymerases **(A)** For 15 cycles, the specified SD and *GoTaq* DNA polymerases were used to amplify a 135 bp artificial DNA template with a hairpin structure. M is a 50-base-pair DNA ladder. **(B)** Amplification of a GC-rich template using PCR. For 30 cycles, a 1.3 kb DNA fragment of the *Mycobacterium tuberculosis* genome (64% GC) was amplified by 1.25, 2.5, and 5 U of SD polymerase (lanes 1–3) and *GoTaq* polymerase (lanes 4–6). M is 1 kb DNA ladder (Ignatov *et al.*, 2014).

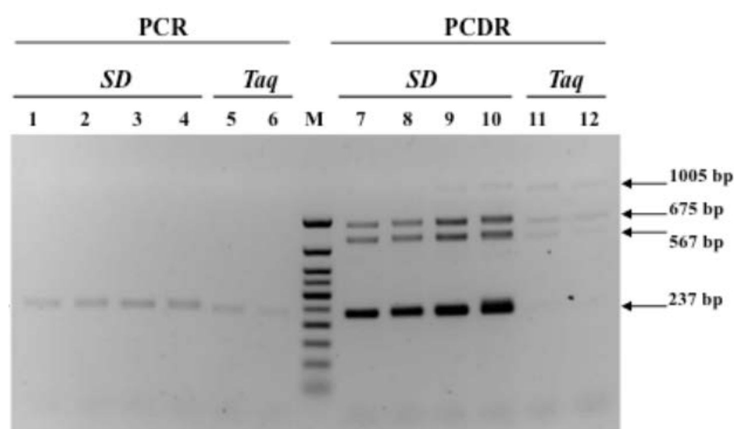


Figure 15. In PCR (lanes 1–6) and PCDR (lanes 7–12) amplifications, 5, 10, 20, or 40 U of SD polymerase (lanes 1–4 and 7–10) or 5 and 10 U of *GoTaq* polymerase (lanes 1–4 and 7–10) were used (lanes 5, 6 and 11, 12). Two primers were used in PCR tests, whereas four primers were used in PCDR assays. Arrows show the locations of the amplicons. M is a ladder of 50 bp (Ignatov *et al.*, 2014).

Quantitative assays compare PCDR to PCR in the same pattern with 20, 200, 2,000, and 20,000 copies of templates. All dilutions examined PCDR with four primers produced the lower Cq values and significantly better fluorescence curves when compared to PCR with two primers (Harris *et al.*, 2013).

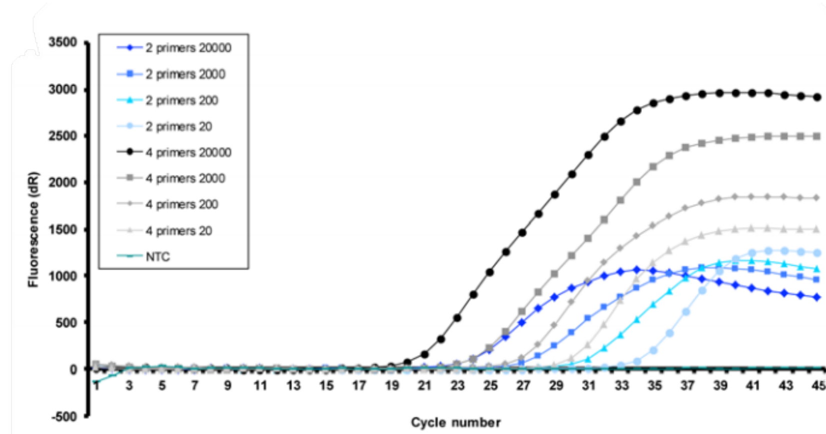


Figure 16. ไม่เห็นเหมือนใน List of figures เลยค่ะIncreased sensitivity of quantitative PCDR when PCR is performed using four primers and two primers PCDR and PCDR master mix were used in amplification processes with 20,000, 2000, 200, or 20 copies template DNA (Harris *et al.*, 2013)

OBJECTIVE

This study aims to study the effectiveness of human leptospirosis detection between real-time polymerase chain displacement reaction (qPCDR) compared to a conventional qPCR technique using two primers based on the *lipL32* gene.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND EQUIPMENTS

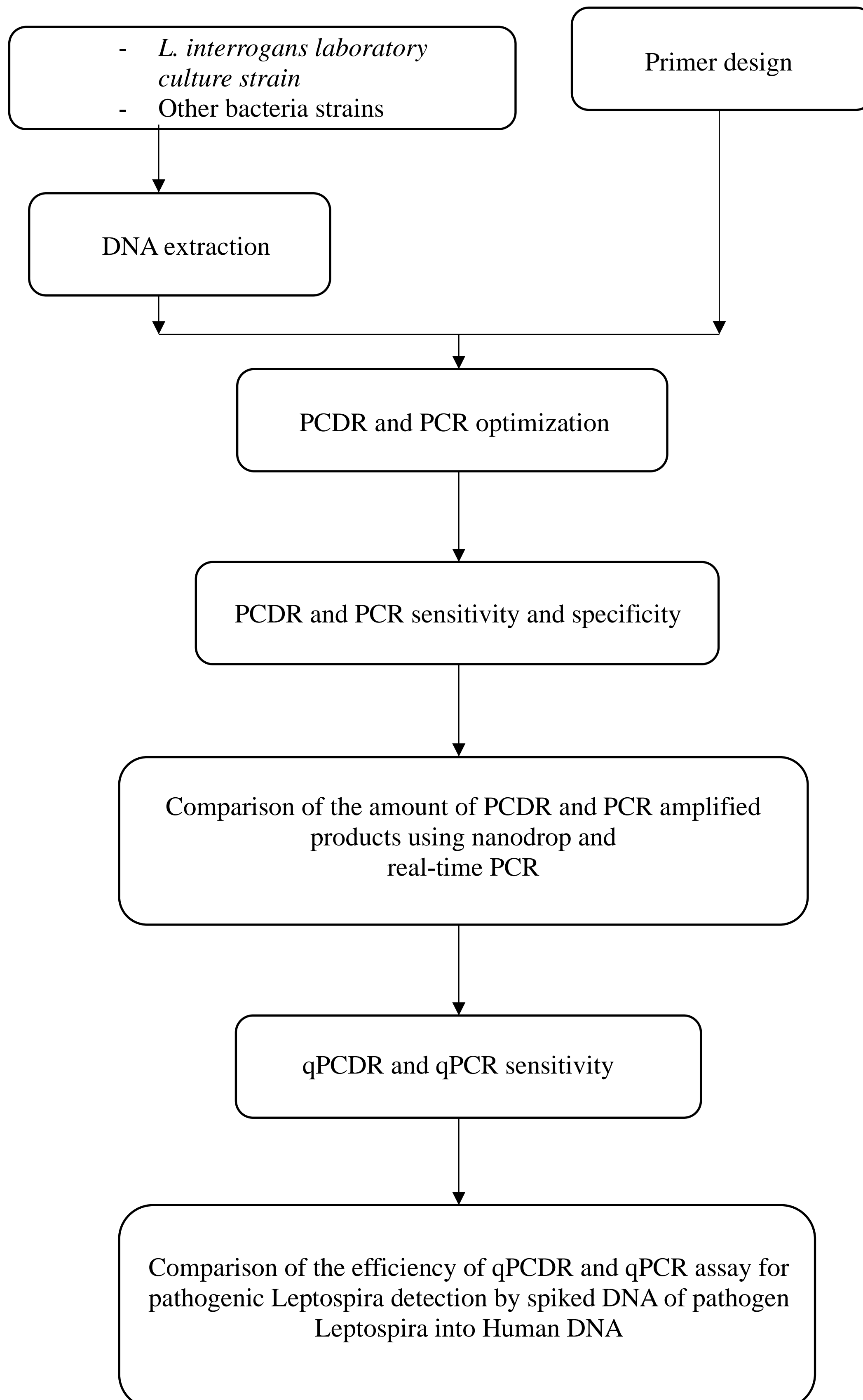
Chemical reagents

Agarose powder	1st BASE, Singapore
WFI Quality water	OmniPur, USA
DNA ladder	Biolab, Germany
dNTPs	Invitrogen, USA
SsoFast Evagreen supermix	Bio-rad, USA
Ethidium bromide	Sigma, USA
Taq DNA Polymerase	Invitrogen, USA
SD DNA Polymerase	Bioron, Germany
QIAamp DNA Blood mini kit	Qiagen, Germany

Equipment and instruments

Autoclave	Tomy, Japan
Balance	Sartorius, Germany
Centrifugation	Four E's Scientific, China
Mini vortex mixture	LabGenius, UK
Hot air oven	Thermo Fisher Scientific, USA
Nanodrop spectrophotometer	Thermo Fisher Scientific, USA
T100 Thermal Cycler	Bio-Rad, USA
LightCycler 480 II	Roche, Switzerland

Flowchart



METHODS

1. Ethics statement

Ethical approval for this study was obtained from the Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University (REC63-157-19-2).

2. DNA samples

Pathogenic *Leptospira* species *(L. interrogans)* laboratory culture strain and non-pathogenic *Leptospira* species *(L. biflexa*) genomic were given by Assoc. Prof. Dr. Direk Limmathurotsakul of the Mahidol Oxford Research Unit at Mahidol University, Faculty of Tropical Medicine in Bangkok, Thailand, and Assoc. Prof. Dr. Thareerat Kalambaheti, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, respectively. Genomic DNA was extracted from laboratory cultures using the Wizard® Genomic DNA extraction kit (Promega, USA).

3. Bacteria strains and DNA extraction

Six pathogenic bacteria and two parasites that are common causes of septicemia or febrile illness were included in this study: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella enterica* serovar typhi*, Burkhoderia pseudomallei, Plasmodium falciparum and Plasmodium vivax.* The bacteria were kindly provided by the technician who works in the Department of Pathology, Faculty of Medicine, Songklanagarind hospital, Hat Yai, Songkhla, Thailand.

The extraction procedure was achieved by the boiling lysis method (Queipo-Ortuno *et al.*, 2008). Boiling was used to produce bacterial isolates. Bacterial cultures were centrifuged for 15 minutes at 15,000×g. The pellet was resuspended in molecular biology grade water (Eppendorf, Hamburg, Germany) and centrifuged at 15,000×g for 10 minutes after the supernatant was removed. The pellet was resuspended in 40 µL of molecular biology grade water, boiled for 10 minutes in a water bath, cooled on ice, and centrifuged for 10 seconds before being kept at -20°C. For PCR, 3 µL aliquots of template DNA were utilized.

4. Human DNA extraction

Human genomic DNA was extracted from 5 ml of whole blood using QIAamp DNA Blood Mini Kit (QIAamp® DNA Mini and Blood) following standard protocols. Briefly, add 20 µL of QIAGEN Protease (or proteinase K) into the bottom of a 1.5 mL microcentrifuge tube and a 200 µL sample was added to the microcentrifuge tube. Following, 200 µL to buffer AL was added to the sample and mixed by pulse-vortexing for 15 seconds, then incubated at 56°C for 10 min. 200 µL of 96-100% ethanol was added to the sample and mixed by pulse-vortexing for 15 seconds. After mixing, the tube was centrifuged for a few minutes to remove the droplet from the lid, and the mixture was transferred to the QIAamp Mini spin column. Close the lid and centrifuge for 1 minute at 6000×g (8000 rpm). The QIAamp Mini spin column

was placed in a clean 2 mL collection tube, and the filtrate tube was discarded. 500 µL of Buffer AW1 was added into the QIAamp Mini spin column and then centrifuged for 1 minute at 6000×g (8000 rpm). The QIAamp Mini spin column was placed in a clean 2 mL collection tube, and the filtrate collection tube was discarded. 500 µL Buffer AW2 was added and centrifuged at full speed 20,000 × g (14,000 rpm) for 3 minutes. The QIAamp Mini spin column was placed in a clean 1.5 mL microcentrifuge tube. 200 µL Buffer AE was added and incubated at room temperature (15–25°C) for 1 minute, and then centrifuge at 6000 × g (8000 rpm) for 1 minute. Human DNA is stored at -20°C until used.

5. Primer design

Primers used in this study were selected based on three criteria; (i) consisting of two pairs of primers, one pair outer primers and one sets of inner primers, (ii) annealing to DNA belonging to pathogenic *Leptospira* spp. based on the conserved sequence of *LipL32* gene but not to intermediate and saprophyte groups, and (iii) targeting the same DNA region. Seventy-seven sequences from pathogenic species carrying *LipL32* gene were downloaded from GenBank for the partial or complete gene (Supplement 1). The number (n) for each species was as follows; *L. borgpetersenii* (n = 16), *L. kirschneri* (n = 6), *L. interrogans* (n = 39), *L. santarosai* (n = 9), *L. weilii* (n = 2), and *L. noguchii* (n = 5). *MuLtiple sequence alignments* were performed *using* the *ClustalW* in BioEdit Version 7.0.4.1. The design of primers was performed by Amplifx software and validated by Primer-Blast. All primer pairs were shown in Table 3, and their relative positions are shown in Figure 17. Primer map accepts a DNA sequence and a textual map showing the annealing positions of PCR primers located on the nucleotides sequence of *L. interrogans* strain RZ11 LipL32 (AF181553.1).

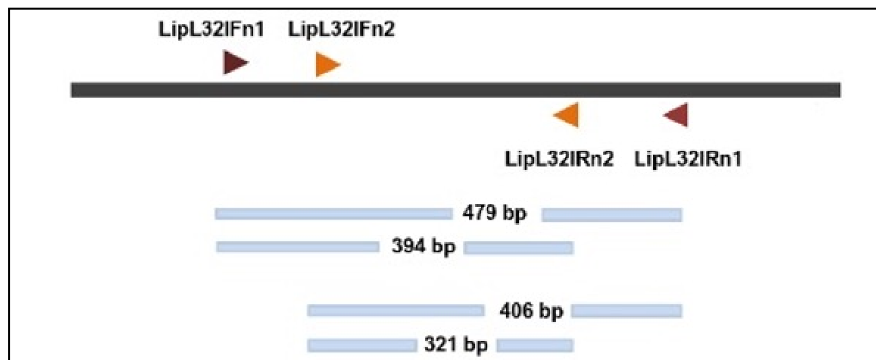


Figure 17. Schematic drawing of the relative four primer positions on *L. interrogans* strain RZ11 LipL32 (AF181553.1).

6. Primer optimization

Primer optimizations were performed both PCDR and PCR amplification. Optimization was used *L. interrogans* as a positive control. The single pair primer that consisting of LipL32IFn1/LipL32IRn1 and LipL32IFn2/LipL32IRn2 were performed in an individual tube.

The PCDR and PCR conditions were optimized by varying 1-3 mM $MgCl_2$ concentration and primer annealing temperature. Gradient PCR was performed in the range of 54 °C to 60 °C based on the melting temperature (T_m) of each primer pair.

PCDR: Twenty-three µL of reaction mixture containing 1x PCR buffer, 1-3 mM $MgCl_2$, 0.25 mM of dNTPs, 0.25 µM of each primer, 0.5U SD polymerase (Bioron GmbH, Germany) and three µL of *L. interrogans* gDNA (Table 4). The PCDR cycling condition was set as follows: initial denaturation at 92°C (2 min), 35 cycles of denaturation at 92°C (30 seconds), annealing varying at 54 °C to 60 °C of each primer pair (30 seconds) and elongation at 68°C (30 seconds). The last cycle was followed by heating at 68°C (30 seconds).

PCR: Twenty-three µL of reaction mixture containing 1x PCR buffer, 1-3 mM $MgCl_2$, 0.25 mM of dNTPs, 0.25 µM of each primer 0.5U of *Taq* DNA polymerase (Invitrogen, California, USA), and three µL of *L. interrogans* gDNA (Table 5). The PCR cycling condition was set as follows: initial denaturation at 95°C (5 min), 35 cycles of denaturation at 94°C (1 min), annealing varying at 54 °C to 60 °C of each primer pair (2 min) and elongation at 72°C (2 min). The last cycle was followed by heating at 72°C (5 min).

Five µL of PCR products mixed with 1 µL of loading dye (Orange G) and were visualized by 2% (w/v) agarose gel electrophoresis at 100 volts for 45 mins with ethidium bromide in a UV transilluminator

Table 4 Sequences of primers used in PCR assays.

Primers	Primer sequences (5' to 3')	Melting temperature (T_m)	Product size (bp)
LipL32IFn1	5'-GGATCTGTGATCAACTATTACGGA -3'	54	479
LipL32IRn1	5'-GAAATTCTGTAAAGACCTCTTAC -3'	49	
LipL32IFn2	5'-AAGCATACTATCTCTATGTTTGG -3'	50	321
LipL32IRn2	5'-TGATTCTAGTAAGAGAGTT -3'	44	

Table 5 Composition of master mix for PCDR amplification

Stock solution	Stock conc.	Final conc. (outer primer)	Final conc. (inner primer)
SD polymerase reaction buffer	10X	1X	1X
dNTP	10 mM	0.25 mM	0.25 mM
$MgCl_2$	100 mM	1 to 3 mM	1 to 3 mM
Primer: LipL32IFn1/LipL32IRn1	2.5 µM	250 nM	250 nM
Primer: LipL32IFn2/LipL32IRn2	2.5 µM	-	250 nM
SD polymerase	10U	0.5U	0.5U
PCR-grade water	-	Up to 20 ul	Up to 20 ul
DNA template	-	3	3
Total	-	23	23

Table 6 Composition of master mix for PCR amplification

Stock solution	Stock conc.	Final conc. (outer primer)	Final conc. (inner primer)
PCR Buffer	10X	1X	1X
dNTP	10 mM	0.25 mM	0.25 mM
$MgCl_2$	50 mM	1 to 3 mM	1 to 3 mM
Primer: LipL32IFn1/LipL32IRn1	2.5 µM	250 nM	250 nM
Primer: LipL32IFn2/LipL32IRn2	2.5 µM	-	250 nM
Taq DNA polymerase	5U	0.5U	0.5U
PCR-grade water	-	Up to 20 ul	Up to 20 ul
DNA template	-	3	3
Total	-	23	23

7. Analytical sensitivity and specificity of PCDR and PCR amplification

The gDNA of *L. interrogans* was prepared by ten-fold dilution with PCR-grade water, from 10 to 10^{-8} ng/µL or $2x10^{10}$ to 10^{-3} gDNA copies/µL (as the size of the genome of *L. interrogans* strain is about 4.6 Mb, 1 genome is ~5 fg. gDNA). The specificity was evaluated by non-target DNA templates from non-pathogen *Leptospira* and other bacterial pathogens that are common causes of febrile illness or septicemia, i.e., *B. pseudomallei, E. coli, K. pneumoniae, S. aureus, P. aeruginosa, S. typhi, P. falciparum,* and *P. vivax.*

PCDR: Twenty-three µL of reaction mixture containing 1x PCR buffer, 3 mM $MgCl_2$, 0.25 mM of dNTPs, 0.25 µM of each primer 0.5U SD polymerase (Bioron GmbH, Germany), and three µL of *L. interrogans* gDNA. The PCDR cycling condition was set as follows: initial denaturation at 92°C (2 min), 35 cycles of denaturation at 92°C (30 seconds), annealing at 60 °C of inner primers (LipL32IF2/LipL32IR2) and outer primers (LipL32IF1/LipL32IR1 (30 seconds) and elongation at 68°C (30 seconds). The last cycle was followed by heating at 68°C (30 seconds) as shown in Table 6.

PCR: Twenty-three µL of reaction mixture containing 1x PCR buffer, 1-3 mM $MgCl_2$, 0.25 mM of dNTPs, 0.25 µM of each primer 0.5U of *Taq* DNA polymerase (Invitrogen, California, USA), and three µL of *L. interrogans* gDNA. The PCR cycling condition was set as follows: initial denaturation at 95°C (5 min), 35 cycles of denaturation at 94°C (1 min), annealing at 60 °C of inner primers (LipL32IF2/LipL32IR2) (2 min) and elongation at 72°C (2 min). The last cycle was followed by heating at 72°C (5 min) as shown in Table 6.

PCDR and PCR products were visualized by 2% (w/v) agarose gel electrophoresis with ethidium bromide in a UV transilluminator. PCR-amplified products were measured nucleic acid concentration by nanodrop.

Table 7 Thermal cycling of PCDR & PCR amplification

Thermal cycling condition	PCDR	PCR
Initial denaturation	92°C, 2 min	95°C, 5 min
Denaturation	92°C, 30 seconds	94°C, 1 min
Annealing	60°C, 30 seconds — 35 cycles	60°C, 2 min — 35 cycles
Extension	68◦C, 30 seconds	72°C, 2 min
Final extension	68◦C, 30 seconds	72°C, 5 min

8. Determination the sensitivity of the amplified product of PCDR *and* PCR using SYBR green

The amplified products of two pairs of primers PCDR and one pair primer PCR were subjected to a 10-fold serial dilution and then used as templates in this experiment. This experiment was used the amplification products from material and method 6. as a template. SYBR Green is a fluorescent DNA binding dye that intercalates into dsDNA, allowing measurement of the amount of amplified product. Evaluation of the sensitivity of pathogen *Leptospira* detection between PCDR and PCR products was compared by the Cp values. Lower Cp values indicate high amounts of the target sample, while higher Cp values mean lower amounts of the target nucleic acid. Prepare the master mix, total reaction volume, 20 µL, containing 1X of SsoFast EvaGreen Supermix (Bio-Rad, HercuLes, CA, USA), 0.25 µM each of two primers: LipL32IFn2 and LipL32IRn2 and two µL of the template (Table 7). The real-time PCR was performed of 5 min of initial denaturation at 95 °C and 35 repeated cycles of 94 °C for 1 min, 60°C for 2 min, extension 72°C for 2 min and final extension 72°C for 5 min (Table 8). All amplifications were performed using the LightCycler®480 system (Roche Diagnostics GmbH, Mannheim, Germany).

Table 8 Composition of master mix for qPCR amplification

EvaGreen Supermix	10
0.25 uM of LipL32IFn2 and LipL32IRn2	2
H_2O	6
template DNA	2

Table 9 Thermal cycling of qPCR amplification

Thermal cycling condition	**qPCR**
Initial denaturation	95°C, 5 min
Denaturation	94°C, 1 min (45 cycles)
Annealing	60°C, 2 min (45 cycles)
Extension	72°C, 2 min (45 cycles)
Final extension	72°C, 5 min

9. Sensitivity analysis of qPCDR and qPCR

The gDNA of *L. interrogans* was prepared by ten-fold dilution with PCR-grade water, from 1 to 10^{-5} ng/µL or $2x10^{5}$-2.0 gDNA copies/µL (as the size of the genome of *L. interrogans* strain is about 4.6 Mb, 1 genome is ~5 fg. gDNA). All dilutions were tested in duplicate, while negative control was included in the run. The composition of master mix and thermal cycling of qPCDR and qPCR are presented in Table 9 and 10.

The sensitivity of the two methods was compared by the Cp values. Lower Cp values imply higher amounts of the target nucleic acid. Testing for sensitivity could be reported in the form of the lowest concentration or limit of detection.

10. Detection of pathogen *Leptospira* DNA diluted with Human DNA

Human DNA was extracted from a five ml blood sample obtained from an individual using the QIAamp DNA mini blood kit. Genomic DNA extracted from *L. interrogans* serovars Autumnalis was spiked into human DNA as diluent by ten-fold serial dilution. The composition of master mix and thermal cycling of qPCDR and qPCR are presented in Table 9 and 10. The sensitivity of qPCDR and qPCR for pathogen *Leptospira* detection was determined as described above.

Table 10 Composition of master mix for qPCDR & qPCR amplification

Stock	Reagent	Final
10x	SD polymerase reaction buffer	1x
100 mM	MgCl2	3 mM
10 mM	dNTP	0.25 mM
2.5 µM	Primer: LipL32IFn1/ LipL32IRn1	0.075
2.5 µM	Primer: LipL32IFn2/LipL32IRn2	0.075
10U	SD polymerase	0.3
20x	SYBR green	0.4x
	H_2O	Up to 20 µL
	DNA	5 µL
Total	25 µL	

Table 11 Thermal cycling of qPCDR and qPCR amplification

EvaGreen Supermix	10
250 nM of LipL32IFn2/ LipL32IRn2	2
H_2O	6
DNA	2

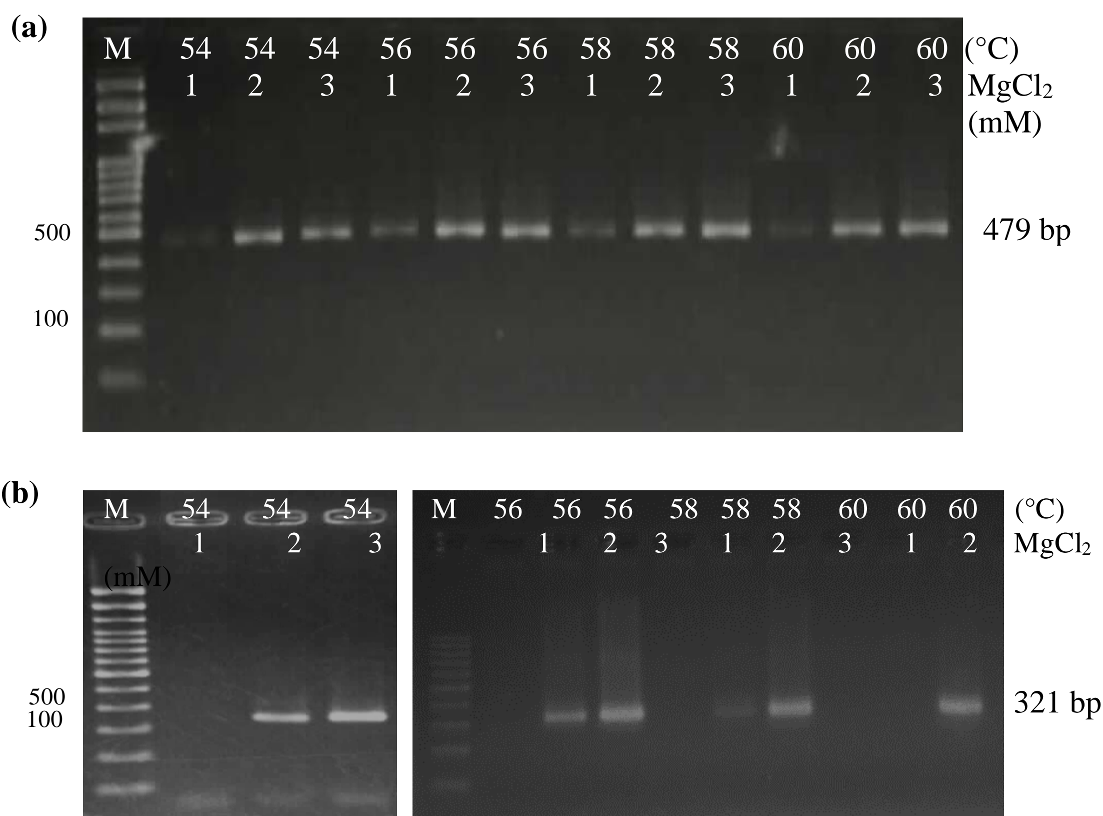
Thermal cycling condition	**qPCDR**	**qPCR**
Initial denaturation	92°C, 2 min	95°C, 5 min
Denaturation	92°C, 30 seconds, 45 cycles	94°C, 1 min, 45 cycles
Annealing	60°C, 30 seconds	60°C, 2 min
Extension	68◦C, 30 seconds	72°C, 2 min

CHAPTER 3
RESULTS

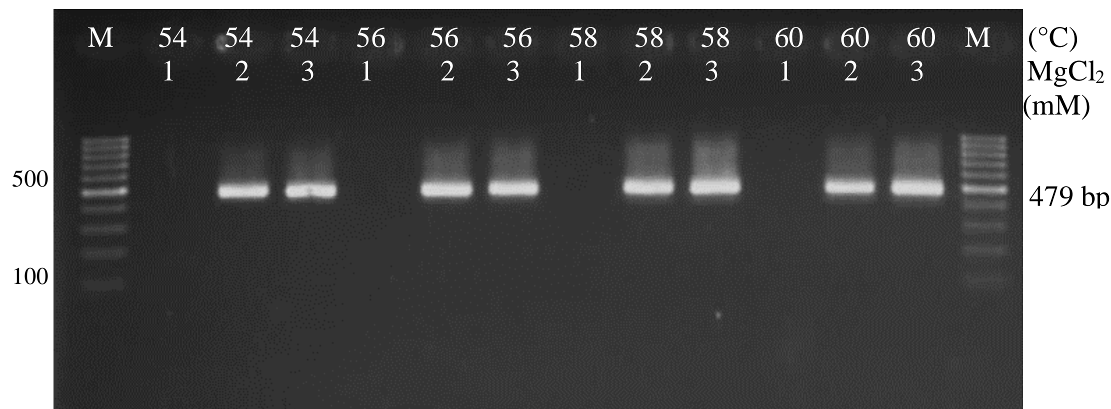
1. Primer optimization

The PCDR and PCR conditions were optimized by varying $MgCl_2$ concentration and primer annealing temperature. Gradient PCR was performed in the range of 54 °C to 60 °C based on the melting temperature (T_m) of each primer pair.

The single pairs primer was used in PCR generated good amplification in annealing temperatures at 60°C with the optimized concentration of $MgCl_2$ 3 mM. At 35 PCR cycles, DNA polymerase produced the predicted amplicons. They showed clearly bands on 2% agarose gel electrophoresis at 100 volts for 45 min. *Taq* DNA polymerase was used in PCR to obtain the PCR product band. LipL32IFn1/LipL32IRn1 showed a 479 bp amplicons (Figure 18a), while the smallest pairs of primers LipL32IFn2/LipL32IRn2 showed a 321 bp amplicons (Figure 18b). SD DNA polymerase was used in PCDR to obtain the PCDR product band. There were 479 (Figure 18c) and 321 bp (Figure 18d), respectively. Moreover, 3 mM $MgCl_2$ selected for the reason that the suitable concentration in PCR and PCDR reaction.



(c)



(d)

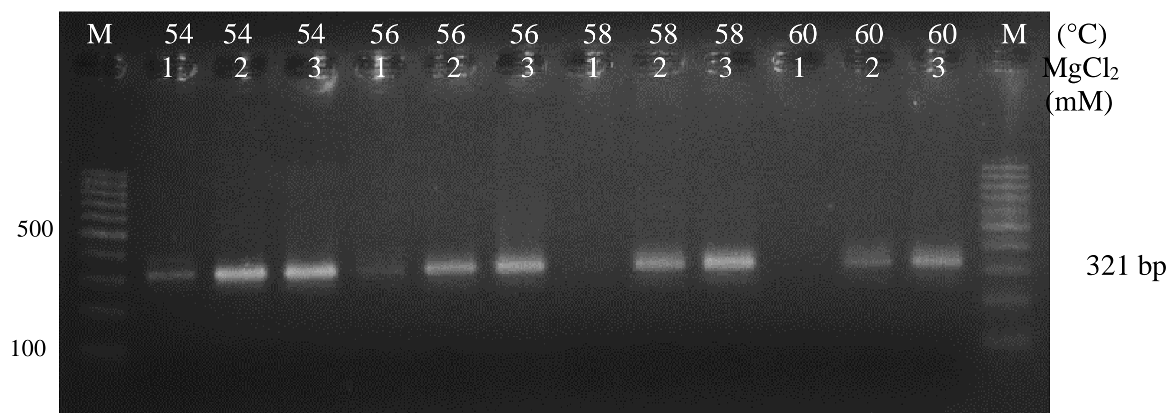


Figure 18. ไม่เห็นเหมือนใน List of figures เลยค่ะ DNA bands of gradient PCDR and PCR experiments captured on 2% agarose gel for each primer pair **(a)** PCR assays carried out with LipL32IF1/LipL32IR1 **(b)** PCR assays carried out with LipL32IF2/LipL32IR2 **(c)** PCDR assays carried out with LipL32IF1/LipL32IR1. **(d)** PCDR assays carried out with LipL32IF2/LipL32IR2. M molecular ladder of 100 bp.

2. Sensitivity and specificity of PCDR and PCR amplification

The gDNA of *L. interrogans* was prepared by ten-fold dilution with PCR-grade water, from 10 to 10^{-8} ng/µL or $2x10^{6}$ to $2x10^{-3}$ gDNA copies/µL (as the size of the genome of *L. interrogans* strain is about 4.6 Mb, 1 genome is ~5 fg. gDNA). In testing for sensitivity, could be reported in form of the lowest concentration or limit of detection (LOD). The amplifications were carried out by PCDR (Figure 19a) and PCR (Figure 19b). LOD detected by two pairs of primers demonstrated that approximately 10^{-3} ng/µL ($2x10^{2}$ gDNA copies/µL) while single pair primer shown the LOD at 10^{-2} ng/µL ($2x10^{3}$ gDNA copies/µL). Apparently, PCDR generated two amplicons size but PCR unable to performed. In addition, all reactions were negative using DNA from non-pathogenic *Leptospira* spp. and one representative each of *B. pseudomallei, E.*

coli, K. pneumoniae, S. aureus, P. aeruginosa, S. enterica serovar typhi*, P. falciparum,* and *P. vivax* (Figure 20).

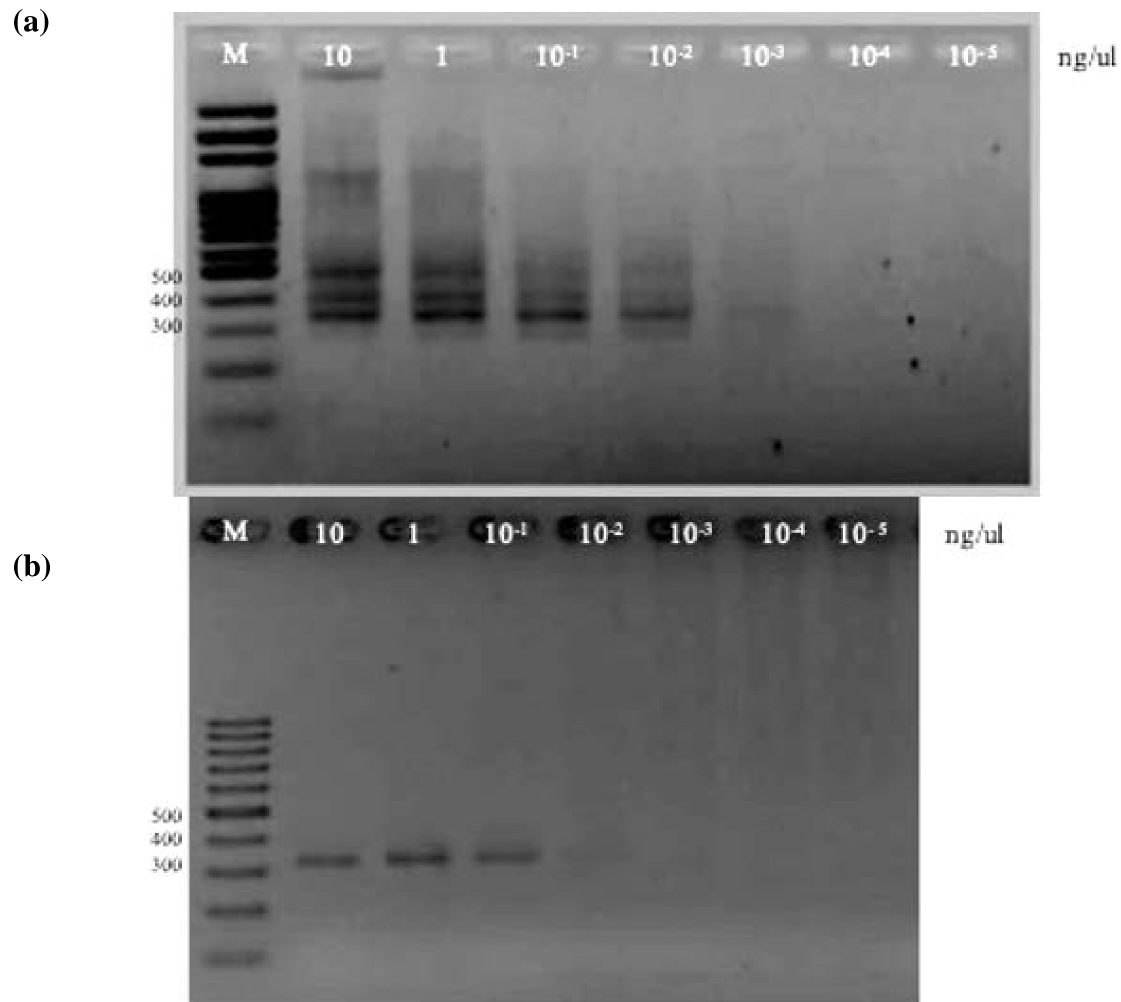


Figure 19. Sensitivity test amplification reactions were carried out using **(a)** PCDR **(b)** PCR and gDNA was prepared by ten-fold dilution with PCR-grade water, from 10 to 10^{-10} ng/μL. M molecular ladder of 100 bp.

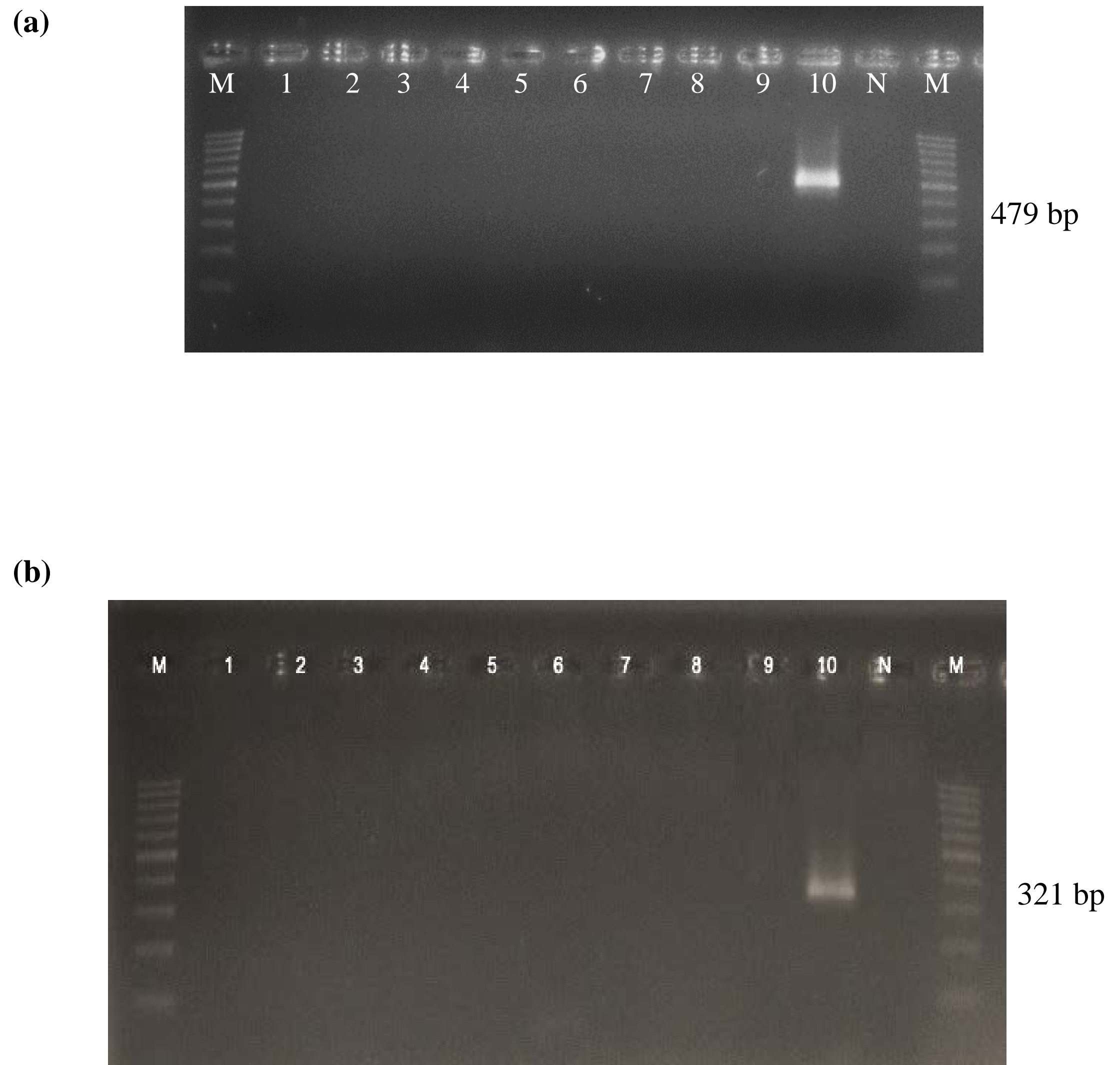


Figure 20. Specificity test. PCR reactions was carried out by non-target DNA templates from non-pathogen *Leptospira* and eight bacterial pathogens, lane 1 to 10 represent for *B. pseudomallei, E. coli, K. pneumonia, S. aureus, P. aeruginosa, S. typhi, P. falciparum, P. vivax.*, non-pathogen leptospira and *L. interrogans*, respectively. Assays contained each primer pair **(a)** for LipL32IF1/LipL32IRn1 and **(b)** for LipL32IFn2/LipL32IRn2. M molecular ladder of 100 bp.

3. Measurement of the nucleic acid concentration and amount of amplicon products from PCDR *and* PCR assay

The amplified products of PCDR and PCR of Figure 20 which started at 10 ng/uL were used as templates.

To compare the nucleic acid concentration between PCDR and PCR products. The result shown that PCDR product (313.4 ng/uL) has higher the nucleic acid concentration than PCR product (228.35 ng/uL) as shown in Figure 21.

To compare the amount of the PCDR and PCR products. This study was used the qPCR method by using SsoFast EvaGreen Supermix (Bio-Rad). The dilution of amplified products stared at 10^2, 10, 1, 10^{-1} and 10^{-2} ng/µL. After the reaction was completed, the mean of the crossing point (Cp) value was evaluated. As shown in Table 12, the Cp value of PCDR lower than that of PCR, implying an the higher the amount of targeted DNA

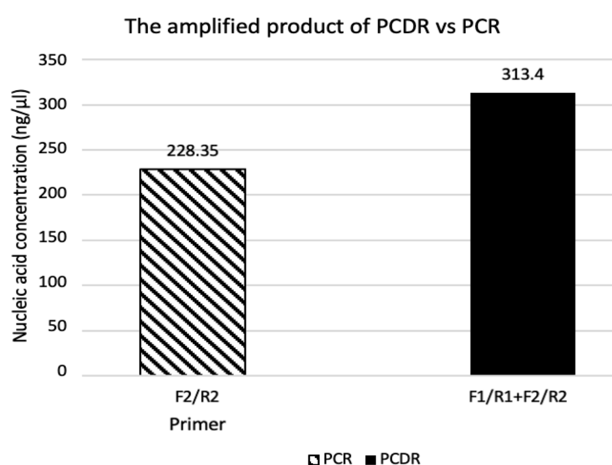


Figure 21. Nanodrop spectrophotometry measurements of amplified PCDR and PCR products

Table 12 The crossing point (Cp) of qPCR and qPCDR

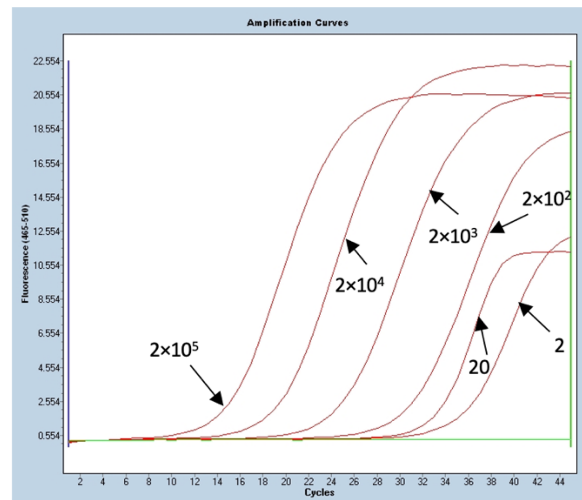
Product conc. (ng/µL)	10^2	10^1	1	10^{-1}	10^{-2}
PCR (F2/R2)	11.125	12.35	12.64	15.89	20.025
PCDR	9.525	9.855	11.94	14.29	18.145

4. Sensitivity analysis of qPCDR and qPCR assays

The gDNA of *L. interrogans* was prepared by ten-fold dilution with PCR-grade water, from 1 to 10^{-5} ng/µL or 2x10^5-2.0 gDNA copies/µL (as the size of the genome of *L. interrogans* strain is about 4.6 Mb, 1 genome is ~5 fg. gDNA). All dilutions were tested in duplicate, while negative control was included in the run.

Under the optimal conditions, qPCDR yielded (Figure 22a) lower Cp values compared with qPCR (Figure 22b). The ΔCp between the two approaches was about 3–5 cycles. The limit of detection of the qPCDR-based approach was 2 copies/µL of *L. interrogans* serovars Autumnalis within 35 cycles, while the qPCR-based approach, the limit was only at 20 copies/µL. Thus, qPCDR provided at least a ten-fold enhancement in sensitivity comparing to qPCR (Table 12).

(a)



(b)

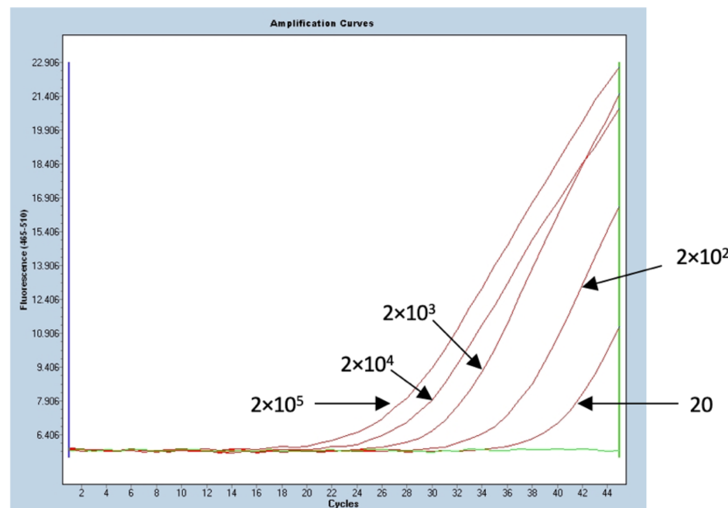


Figure 22. Sensitivity analysis of **(a)** qPCDR and **(b)** qPCR assays for DNA of *L. interrogans* detection by ten-fold dilution with PCR-grade water.

5. Detection of pathogen *leptospira* DNA diluted with human DNA

gDNA of *L. interrogans* serovars Autumnalis was diluted with human DNA by ten-fold serial dilution. The concentration of gDNA was prepared in the range of $2x10^5$ to 2 gDNA copies/μL. In this study, qPCDR yielded (Figure 23a) lower Cp values compared with qPCR (Figure 23b). The ΔCp between the two approaches was about 6–7 cycles. The limit of detection of the qPCDR-based approach was $2x10^3$ copies/μL of *L. interrogans* serovars Autumnalis within 36 cycles, while the qPCR-based approach, the limit was only at $2x10^4$ copies/μL within 36 cycles, Table 12. Thus, qPCDR provided at least a ten-fold enhancement in sensitivity comparing to qPCR.

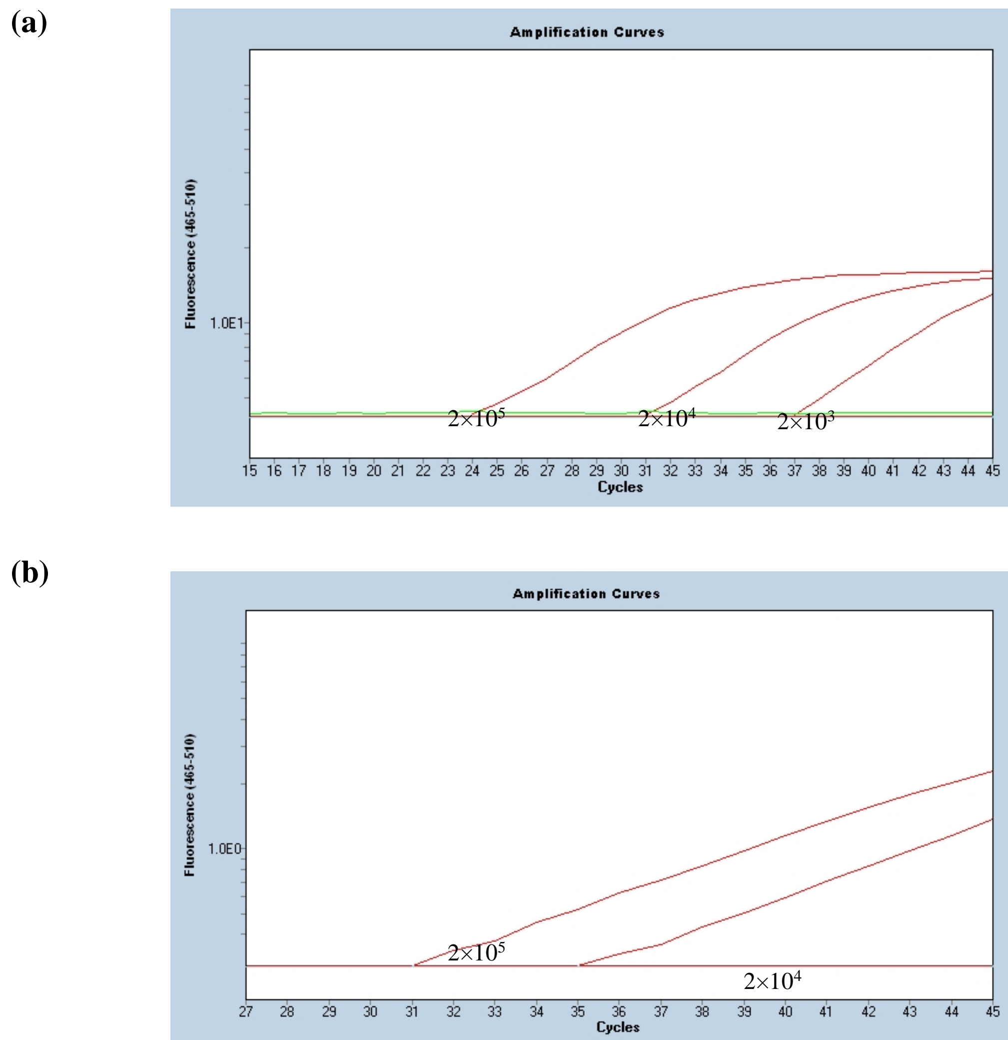


Figure 23. Evaluation of **(a)** qPCDR and **(b)** qPCR assays for DNA of *L. interrogans* detection by ten-fold dilution with human DNA.

Table 13 Cp of 10-fold serial dilutions of *L. interrogans* serovars Autumnalis of qPCR and qPCDR amplification NA, no amplification.

Dilution (copies/uL)	$2x10^5$	$2x10^4$	$2x10^3$	$2x10^2$	20	2
L. interrogans serovars Autumnalis diluted in PCR-grade water						
Cp of qPCR	19.86	22.18	26.07	31.24	35.39	NA
Cp of qPCDR	14.15	18.81	23.74	29.61	32.66	35.48
Δ Cp	5.71	3.37	2.33	1.63	2.73	-
L. interrogans serovars Autumnalis diluted with human DNA samples						
Cp of qPCR	30.95	34.97	NA	NA	NA	NA
Cp of qPCDR	23.48	30.76	36.22	NA	NA	NA
Δ Cp	7.47	4.21	-	-	-	-

CHAPTER 4

DISCUSSION

The influence of annealing temperature to study characterizes the genotyping pattern of single and four oligonucleotide primers focusing on *lipL32* gene with PCR and PCDR, tested at the different temperature ranges. At 35 cycles of PCR and a temperature of 60 °C, the primers produced the predicted amplicons. The optimum concentration of $MgCl_2$ for each primer was independent of the GC/AT ratio of the primer and the number of DNA bands amplified (Park and Kohel, 1994) . In this study was 3 mM. Excessive of $MgCl_2$ facilitate non-specific binding of primer with template DNA which results in non-specific DNA bands in agarose gel electrophoresis.

PCR and PCDR have the potential to be useful in diagnosing leptospirosis, by the reason of its high specificity and sensitivity, with capability of detecting as few as 10 microorganisms in a clinical samples and able to detect them during the first 10 days of the disease before clinical expression (Brown *et al.*, 1995) (Merien *et al.*, 1992). Principally, when specific pathogens that are difficult to culture *in vitro* or require a long cultivation period are expected to be present in specimens (Yamamoto, 2002). Early diagnosis of human leptospirosis is always desirable, due to Its symptoms are similar to those of a variety of other diseases, including influenza, meningitis, hepatitis, dengue fever, and other viral hemorrhagic fevers (Scarcelli *et al.*, 2003). Therefore, specificity is the indispensable. To estimate the validity of PCDR and PCR for the detection of pathogenic *leptospira* spp. in clinical samples. Eight laboratories participated bacterial isolation were tested. The primers incapable to amplified participated bacterial isolation excepting gDNA of pathogenic *leptospira* with both PCDR and PCR product was generated.

gDNA diluted by serial ten-fold dilution either molecular biology-grade water and human DNA. PCR assays contained one and two pairs of primer. In testing for sensitivity, could be reported in form of the lowest concentration or limit of detection (LOD). The amplifications were carried out by PCDR and PCR. LOD detected by two pairs of primers demonstrated that approximately 10^{-3} ng/µL while single pair primer shown the LOD at 10^{-2} ng/µL. Apparently, PCDR generated two amplicons size but PCR unable to performed. The differences in sensitivity between PCDR and PCR presented to be due to differences in the efficiency of the individual amplification. These differences could not be explained by differences in product sizes (Durigon *et al.*, 1993). Conventional PCR tests were unable to be thoroughly examined, leaving their diagnostic usefulness in issue (Brown *et al.*, 2003), it was followed by real-time quantitative PCR (qPCR), which combines amplification and detection of amplified product in the same procedure, with high sensitivity and specificity, and also a low risk of contamination. (Espy *et al.*, 2006). qPCR also has several usefulness over conventional PCR, uncomplicated to execute and less time consuming, facilitates online monitoring (Ahmed *et al.*, 2009) (Picardeau *et al.*, 2014). Furthermore, Cp-values were used to compare the result of different qPCR procedures.

In the present study, four-primer qPCDR for the detection of pathogenic *Leptospira* was used. The application of four-primer qPCDR enabled the detection of the target pathogenic DNA concentration ten-fold lower than that was required by qPCR under similar conditions. As the

result of sensitivity, four-primer qPCDR could be detection the concentration of pathogenic *Leptospira* at 2 copies/uL, while qPCR was 20 copies/uL. In addition, four-primer qPCDR could be detected of pathogen *leptospira* DNA diluted with human DNA ten-fold lower than that qPCR. The Cp values were decreased by four to seven cycles in four-primer qPCDR compared with qPCR, implying a shorter reaction time needed by qPCDR**.** SD polymerase employed for the amplification of the target DNA is a DNA polymerase with 5′ to 3′ strand displacement activity and lacking exonuclease activity. This polymerase has been successfully applied to DNA amplification techniques, e.g., isothermal DNA amplification, conventional PCR and PCDR.

CHAPTER 5

CONCLUSION

The performance evaluation and diagnostic accuracy assessment of the qPCDR and qPCR procedures are described in this study, with the goal of clinical validation for early detection of human leptospirosis. When comparing SD DNA polymerase to *Taq* DNA polymerase. The test is technically reliable for detecting pathogenic leptospiral DNA in clinical samples, with a superior performance when compared to SD DNA polymerase. SD polymerase is more efficient since the test may be performed in less time than a *Taq* polymerase-based assay. We also showed that decreased Cp levels found in qPCDR may be used to detect pathogenic *Leptospires.*

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SUPPLEMENT

Supplement 1 Accession number of pathogenic *leptospira* spp. used in primers design.

Accession number	Pathogenic strains
AY442332.1	*L. interrogans* serovar hardjo
AY609332.1	*L. interrogans* serovar Wolffi
AF245281.1	*L. interrogans* putative
AY609329.1	*L. interrogans* serovar Paidjan
AY609328.1	*L. interrogans* serovar Hebdomadis
AY568679.1	*L. interrogans* strain Lai
KC800990.1	*L. interrogans* serovar Canicola strain RTCC 2824
EU871716.1	*L. interrogans* serovar Pomona
JN831363.1	*L. interrogans* serovar Canicola strain RTCC 2805
AY461908.1	*L. interrogans* strain 56601
AJ580493.1	*L. interrogans* serovar canicola
AY609323.1	*L. interrogans* serovar Pyrogenes
HM026175.1	*L. interrogans* serovar Canicola immunodominant
EU871723.1	*L. interrogans* serovar Grippotyphosa strain Moskva V
AY461905.1	*L. interrogans* strain Hardjoprajitno
AY461907.1	*L. interrogans* strain L1-130
EU871718.1	*L. interrogans* strain UPM-ND
AY461904.1	*L. interrogans* strain Hond Utrecht
AY461910.1	*L. interrogans* strain Pomona RZ11
AY461901.1	*L. interrogans* strain Jez-Bratislava
JQ013518.1	*L. interrogans* isolate F7
GU220823.1	*L. interrogans* serovar Hebdomadis
JQ013520.1	*L. interrogans* serovar Balico
JQ013519.1	*L. interrogans* serovar Manilae
DQ149595.1	*L. interrogans* serovar Sejroe
AY461903.1	*L. interrogans* strain Van Tienen
AY461902.1	*L. interrogans* strain Akiyami
AY461906.1	*L. interrogans* strain Kremastos
DQ092412.1	*L. interrogans* serovar Canicola
JN210551.1	*L. interrogans* serovar Autumnalis strain N2
KC800991.1	*L. interrogans* serovar Hardjo strain RTCC 2810
AB094433.2	*L. interrogans* serovar icterohaemorrhagiae
AB094434.2	*L. interrogans* serovar canicola
KC800993.1	*L. interrogans* serovar Icterohaemorrhagiae strain RTCC 2812
KC800987.1	*L. interrogans* serovar Pomona strain RTCC 2822
KC800988.1	*L. interrogans* serovar Pomona strain RTCC 2815

AY776293.1	*L. interrogans* serovar Pomona strain Luo
AF181553.1	*L. interrogans strain RZ11*
KP032210.1	*L. interrogans* strain N2
AY609333.1	*L. borgpetersenii* serovar Mini
AY568680.1	*L. borgpetersenii* strain M10
EU871722.1	*L. borgpetersenii* strain UPM-R48
AY461893.1	*L. borgpetersenii* strain 1409/69
AF181554.1	*L. borgpetersenii* strain 203
AY461895.1	*L. borgpetersenii* strain 93U
AY461898.1	*L. borgpetersenii* strain Piyasena
AY461894.1	*L. borgpetersenii* strain Mus 127
AY609333.1	*L. borgpetersenii* serovar Mini
EU526389.1	*L. borgpetersenii* serovar Javanica strain R1R
EU526390.1	*L. borgpetersenii* serovar Javanica strain R1L
EU871722.1	*L. borgpetersenii* strain UPM-R48
AY568680.1	*L. borgpetersenii* strain M10
AY461897.1	*L. borgpetersenii* strain Sari
AY461896.1	*L. borgpetersenii* strain Nona
AY461900.1	*L. borgpetersenii strain Veldrat Batavia 46*
AY461917.1	*L. kirschneri* strain 5621
AY461912.1	*L. kirschneri* strain Erinaceus Auritus 670
AY461911.1	*L. kirschneri* strain Musa
AY461915.1	*L. kirschneri* strain RM52
AY461914.1	*L. kirschneri* strain LT1014
AY461916.1	*L. kirschneri* strain Kambale
AY609331.1	*L. weilii* serovar Manhao II
AY461930.1	*L. weilii* strain LT89-68
AY461923.1	*L. santarosai* strain LT117
AY461921.1	*L. santarosai* strain MR12
AY461924.1	*L. santarosai* strain LT1098
KJ152438.2	*L. santarosai* strain UW
AY461927.1	*L. santarosai* strain LT821
AY461928.1	*L. santarosai* strain LT79
AY461922.1	*L. santarosai* strain CZ288
AY461925.1	*L. santarosai* strain CZ299
AF181555.1	*L. santarosai* strain CA299
AY461918.1	*L. noguchii* strain 1011
AY461920.1	*L. noguchii* strain LT796
AY461919.1	*L. noguchii* strain LSU2580
AF181556.1	*L. noguchii* strain Fort Bragg
AY609326.1	*L. noguchii* serovar Pomona

Vitae

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